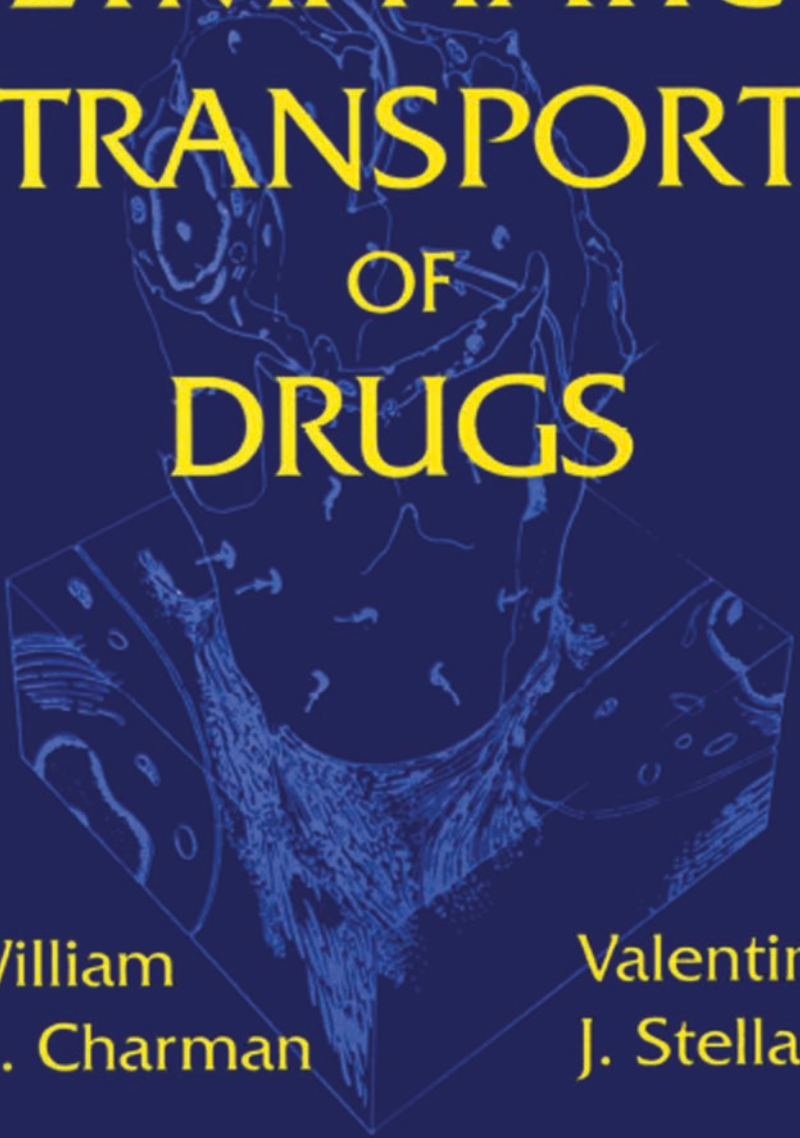
LYMPHATIC TRANSPORT OF DRUGS



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LYMPHATIC TRANSPORT OF DRUGS

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INTRODUCTION

The determinants of the lymphatic transport of lipophilic and polar drugs after oral or parenteral administration have not been extensively reviewed *per se*, although numerous papers and books have addressed the issues relating to the absorption and transport of dietary lipids, lipophilic xenobiotics, and the lipid soluble vitamins after oral administration. References to these books and papers appear throughout the various chapters in this book. In the early 1980s we, along with our colleague Dr. Tetsuo Noguchi, began to explore the factors governing the relative mesenteric lymph or portal blood transport of a number of xenobitics, drugs, and prodrugs after oral administration. As our studies progressed, we began to realize the misunderstandings often apparent within the pharmaceutical sciences with regard to lymphatic drug transport. We therefore thought it appropriate to prepare a book with the primary objective to review and clarify those determinants which affect, and those which can be manipulated to control, the uptake and delivery of drugs and xenobiotics to the lymph. The principle audience for this book is the pharmaceutical scientist interested in designing new agents for delivery to the lymph, or those individuals attempting to alter the delivery of current therapeutic agents through either formulation or prodrug approaches. A concerted effort was made when organizing this book to be as comprehensive as possible, while still maintaining a focus on the "drug delivery" aspects. It is hoped that the material presented will stimulate greater interest and appropriate research into those factors affecting the lymphatic transport of drug molecules.

The book is divided into nine chapters. The first chapter focuses on the anatomy and physiology of the lymphatics, a subject area that is often poorly appreciated by pharmaceutical scientists. The majority of the published data on the lymphatic transport of drugs and xenobiotics has considered intestinal lymphatic transport after oral administration in various metabolizable lipid vehicles such as long chain triglycerides. Therefore, to fully understand the manner in which pharmaceutical vehicles may alter intestinal lymphatic transport, the second chapter reviews the physicochemical and biochemical events involved in the intestinal processing of dietary lipids.

One of the confusing aspects which becomes apparent when reviewing published work and comparing results across studies are the different methodologies employed in lymphatic transport studies. For example, the animal species employed, the fasting state of the animals, the site(s) of lymph vessel cannulation, whether lymph concentration or cumulative amount transported in the lymph are reported etc., have made comparisons between studies very difficult. Additionally, most reported lymph transport studies have not performed what we call mass balance experiments which enable determination as to whether an experimental variable affected only lymph transport, or whether manipulation of the particular variable affected the amount of drug reaching the systemic circulation (i.e., bioavailability enhancement). This, and various other different methodologies for assessing intestinal lymphatic transport, are

reviewed in the third chapter. Also included in this chapter is a brief review of methods for characterizing intestinal lipoproteins, and some morphological methods for assessing intestinal transport pathways. We believe that integrated approaches such as those described in the third chapter are necessary to comprehensively evaluate intestinal lymphatic drug transport.

Chapter 4 focuses on lipid vehicle and formulation effects on intestinal lymphatic drug transport. This chapter not only reviews the known literature on the intestinal transport of lipophilic molecules, but attempts to rationalize how (i) transport is affected by the vehicle in which the drug/xenobiotic is administered, and (ii) the transport of lipophilic drug molecules may be coupled to the simultaneous processing of the vehicle and its metabolic breakdown products. An important section of this chapter is that which deals with the predictability of lymph transport i.e., what are the important physicochemical and metabolic properties of drugs and xenobiotics, and what are the necessary characteristics of the coadministered vehicles, which would allow us to predict, *a priori*, whether a drug is a potential candidate for lymphatic transport after oral dosing. This leads into chapter 5 which reviews examples (both positive and negative) where the physicochemical and metabolic properties of drugs have been changed via prodrug manipulations to promote lymphatic transport. An important conclusion in this chapter is the need to balance the metabolic stability of the prodrug within the intestinal lumen and enterocyte to its ability to revert to the parent drug once released into systemic circulation. This represents a considerable challenge to the prodrug scientist.

Assuming that a drug/xenobiotic is transported to the systemic circulation via the intestinal lymph in association with lipoproteins, how is the agent then released from the lipoprotein? This subject is reviewed in the sixth chapter. A common misconception is that lipophilic drugs transported in the lymph in association with chylomicrons will only be released by the metabolic processing and systemic distribution of the metabolic fragments of chylomicrons and lipoproteins. The importance of diffusional release of the drug, due to the large surface presented by the chylomicrons when they enter the systemic circulation, is not generally appreciated.

Chapters 2 through 6 are largely centered on factors affecting the transport and delivery of lipophilic drugs via the lymph after oral administration. Considerable interest has recently arisen regarding the factors which affect the transport of polar drugs and macromolecules from the intestinal tract into systemic circulation via the lymph. Chapter 7 is devoted to describing the lymphatic transport of polar drugs and macromolecules after gastrointestinal dosing.

Recent research into the transport of drugs into the lymph after parenteral administration is reviewed in chapter 8. There is considerable interest in this research area since the lymphatics are a major site of metastases, and it has been shown that selective delivery of chemotherapeutic agents to the lymphatics might have significant clinical benefits in the treatment of cancer. The

treatment of immunological disorders, bacterial and viral infections might also benefit from selective lymph delivery.

The last chapter in this book describes the role that particulate drug delivery systems might play in lymph delivery. This is currently a very controversial area of drug delivery research. It is hoped that this review will help clarify the current status of this area of drug delivery research, especially as it relates to delivery of agents to the lymph.

The lymphatic transport of drugs after oral and parenteral administration is an exciting area of research. It was our intention to provide an up-to-date review of the subject through this book. The coverage of the chapters in this book reflects our views as editors, and therefore, is limited by the our biases and experiences, which we hope the reader will forgive.

Finally, we would like to express our sincere appreciation to the authors of the various invited chapters for providing their manuscripts in a timely manner and who responded positively to our initial critiques. The University of Kansas provided a sabbatical leave for V.J.S. to the Victorian College of Pharmacy to allow him to complete his chapter and help edit the initial drafts of the various manuscripts; the Center for Drug Delivery Research provided travel support for W.N.C. to the University of Kansas to help finalize the editing; the National Institute of Health (GM-33387) and R.P. Scherer Corporation provided grant monies that supported research by the editors in the area of lymphatic drug delivery. We would also like to thank Miklos Lipcsey for his help with the figures and Dr. Susan Charman for her valuable critique of the book and its various chapters. Finally, we would like to express our appreciation to Susan and Beth for their patience and encouragement.

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Among other awards, he has received the Dolph Simons Award for Biomedical Research and the H.O.P.E. award for excellence in teaching, both from the University of Kansas, and has twice won the Lederle Award.

Dr. Stella has presented more than 55 invited lectures at international meetings, 30 invited lectures at national meetings, and approximately 32 guest lectures at universities and institutes. He has published more than 95 research papers, 16 reviews and book chapters, 4 books, 32 professional and continuing education articles, and 89 abstracts; and he holds 10 patents. Dr. Stella's major research interests are in the area of the application of physical/organic chemistry to the study of factors affecting drug delivery. In particular he is working in the area of chemical kinetics, preformulation workup of cytotoxic drugs, improved drug delivery through the use of prodrugs, and the mechanism of lymphatic drug delivery.

To Susan and Beth

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Chapter 1

ANATOMY AND PHYSIOLOGY OF THE LYMPHATICS

Caitriona M. O' Driscoll

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I. INTRODUCTION

The major physiological function of the lymphatic system is to maintain the body's water balance; thus, it acts as a drainage system throughout the body returning excess fluid, protein, and waste products from the tissue space into the blood circulatory system.[1] In addition, certain lipophilic compounds including long chain fatty acids, triglycerides, cholesterol esters,[2] lipid soluble vitamins,[3] and some xenobiotics, including DDT (2,2-bis(*p*-chlorophenyl)1,1,1-trichloroethane; *p,p*-DDT),[4] are transported via the lymphatic system after peroral administration. Due to its unique anatomy and physiology, the potential exists to exploit the lymphatic system as a means of drug delivery.[5–9] The aim of this chapter is to describe the anatomy and physiology of the lymphatic system, as a detailed knowledge of this area will facilitate maximum utilization of this route for drug delivery. In addition, this knowledge will facilitate the design of optimum drug delivery systems which have physicochemical properties complimentary to the physiological constraints of lymphatic drug delivery.

II. ANATOMY OF THE LYMPHATIC SYSTEM

A. GENERAL STRUCTURE

The lymphatic system consists of a network of vessels (lymphatics), which drain most regions of the body. Lymph is the fluid carried within these vessels. Figure 1 shows the location of the principal components of the lymphatic system in humans.[10] There are numerous lymph nodes along the lymphatic route and there is also significant branching in the network with vessels ranging from 10 μm (lymphatic capillaries) to 2 mm (thoracic duct) in diameter.[11] It should be noted that this anatomical arrangement (Figure 1) is subject to significant interspecies variation and, in some cases, individual variation within a species.[1]

Lymphatics are distributed throughout the body, i.e., wherever lymph is formed. Lymph originates in interstitial fluid which in turn is derived from plasma exudate; consequently, lymphatics are only found in tissues that possess blood vessels.[12] No lymphatics are, therefore, found in cartilage or in the optic cornea and lens. However, if the cornea becomes damaged and vascularized, lymphatics will follow the distribution of the blood vessels.[13] It has traditionally been accepted that lymphatics are absent from the central nervous system since blood vessels in this area are believed to restrict the loss of plasma proteins from their lumen, consequently eliminating the need for lymphatic drainage. Recently, however, it has been suggested that although no discrete lymphatics are present in the brain and spinal cord some lymphatic drainage may occur via prelymphatic pathways lying within the sheaths of arteries running from the brain and spinal cord.[12] Alternatively, the cerebrospinal fluid may be partly drained via lymph.[14] Lymphatics are also absent from the bone marrow, which is possibly due to the high permeability of the marrow sinusoids which may eliminate the necessity for lymph formation.[12]

The lymphatic system is designed and structured such that fluid, i.e., lymph, drained from the interstitial space is returned via the lymphatics into the blood system. The nature and size of the lymphatic vessels vary along the route. The most peripheral and smallest components of the system are termed lymphatic capillaries, also referred to as "terminal" or "initial" lymphatics. The capillaries unite to form collecting vessels. Those which carry lymph to the regional nodes are termed prenodal collecting lymphatics. Postnodal collecting vessels carry the lymph away from the nodes to larger vessels or between successive sets of nodes. The routes taken by these vessels generally follow those of the corresponding blood vessels. The term "trunk" is used to describe the larger vessels which drain lymph from the the final set of nodes into the ducts. The lymphatics from the intestinal, hepatic, and lumbar areas drain into the cisterna chyli, which is a sac-like structure at the distal end of the thoracic duct. The latter ascends from the cisterna chyli and along its route receives lymph from the mediastinum and eventually carries the major portion of the body lymph. The thoracic duct enters the lower region of the chest by passing through the aortic opening of the diaphragm. It runs upwards with the aorta to its left and the azygos vein to its right. At the fifth thoracic vertebra it crosses to the left side of the body and

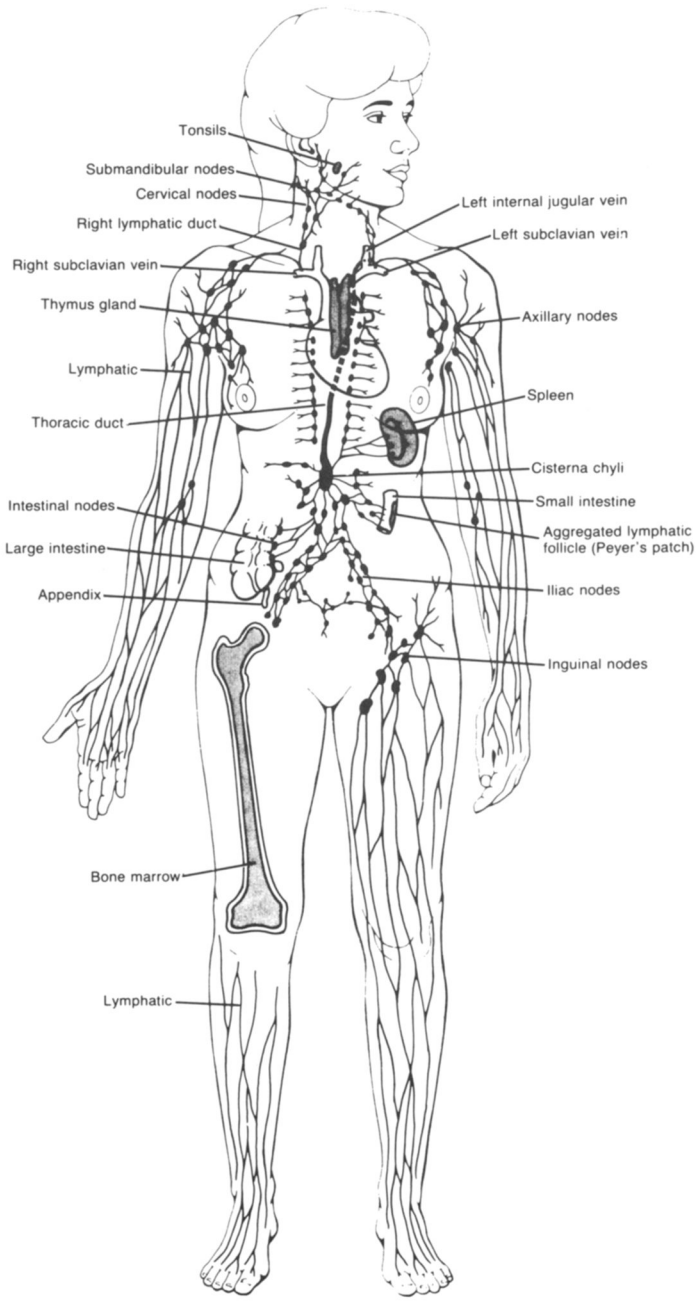

FIGURE 1. Location of the principal components of the lymphatic system. (From Tortora, G. J., in *Principles of Human Anatomy,* 5th. ed., Harper & Row, New York, 1989, chap. 15. With permission.)

continues along the left side of the esophagus. In the neck it arches to the left and descends to join the blood system at the junction of the jugular and the left subclavian veins. Just prior to this junction the thoracic duct may receive lymphatics from the thorax, upper limb, head, and neck. Another duct termed the right lymphatic duct sometimes exists. It is not more than a centimeter long and carries lymph from the lymphatics in the upper right segment of the body. It enters the blood circulation on the right side of the neck. If this duct is missing, the contributing lymphatics drain independently into the junction of the right subclavian and internal jugular veins.[12]

B. LYMPHATIC CAPILLARIES

The lymphatic capillaries form an extensive network which absorbs lymph from the interstitial spaces. The distal capillaries are closed at their peripheral ends. The vessels vary in diameter from 10 to 60 μm.[11,15] The capillary walls are thin (50 to 100 nm) and are made up of a single layer of nonfenestrated endothelial cells with an incomplete basal lamina. The nucleus of each cell is located in the center of the cell and, characteristically, projects into the lumen (Figure 2).[16] Invaginations (caveoli or pinocytotic vesicles) are seen on both luminal and abluminal surfaces. The usual organelles including mitochondria, rough endoplasmic reticulum, a Golgi apparatus, a pair of centrioles, lysosomes, and endocytotic vesicles are found within the cells.

Electron microscopy has enabled the distinguishing properties of the lymphatic capillaries to be detected. They are as follows: (i) the lymphatic capillaries are usually much bigger than blood capillaries, they have irregular walls, and are often collapsed; (ii) the endothelial cells lack a continuous basal lamina; (iii) numerous cytoplasmic projections extend from both luminal and abluminal surfaces to give an irregular shape compared to blood capillaries; (iv) lymphatic capillaries are held open by anchoring filaments; and, (v) neighboring endothelial cells overlap and "open" intercellular junctions are formed.[17]

In the lymphatic endothelial capillaries of the lung, numerous intracytoplasmic microfilaments with actin-like properties have been identified.[18] These filaments may function as a contractile system to regulate the separation of endothelial intercellular junctions. In most species the lymphatic capillaries do not have smooth muscle cells and, consequently, are not intrinsically contractile.[19]

C. INTERCELLULAR JUNCTIONS

Three types of lymphatic endothelial cell contacts have been identified and these are where the endothelial cells (a) touch by abutment of their cytoplasm, (b) overlap to varying degrees and, (c) interdigitate.[20] The distance between two adjacent cells is in dispute as estimates ranging between several microns[21–23] and 15 to 20 nm[24–26] have been reported. In some cases of overlapping cells, the gap extends the entire length of the intercellular junction (Figure 3).[27] These intercellular junctions are a unique property of lymphatic capillaries and are very important features in the transport of fluid and macromolecules into the lym-

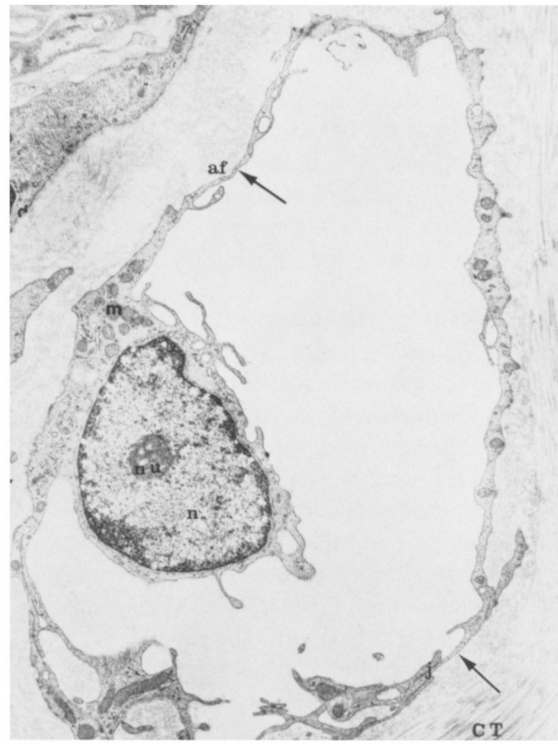

FIGURE 2. A survey electron micrograph illustrating the lymphatic capillary in cross section. The close association of the adjoining connective tissue components (CT) with the lymphatic wall is maintained by numerous anchoring filaments (af) which appear as a meshwork of fine filaments. The endothelium is extremely attenuated at various points (arrows), and the nucleus (n) with its nucleolus (nu) protrudes into the lumen. Several intercellular junctions (j) are observed. Mitochondria (m) appear in the juxtanuclear region as well as in the thin cytoplasmic rims. (Magnification × 11,000.) (From Leak, L. V., *Microvasc. Res.*, 2, 361, 1970. With permission.)

phatic lumen. In some situations the apposing membranes are held together by junctional complexes such as fasciae adherentes (intermediate junctions) which reduce the distance between the cells to 10 to 25 nm, or by fasciae occludentes (tight junctions) which seal the intercellular junction, thereby preventing the passage of molecules such as lanthanum and peroxidase.[27] However, in most instances, these junctional complexes do not appear to encircle the cells, but instead exist along a segment of their borders.[20] The intercellular junctions open when the pressure in the interstitial fluid exceeds that within the lymphatic capillaries.

D. ANCHORING FILAMENTS

The lymphatic capillary wall lacks a continuous basement membrane; consequently, it is fragile and in danger of being crushed. To avoid this problem,

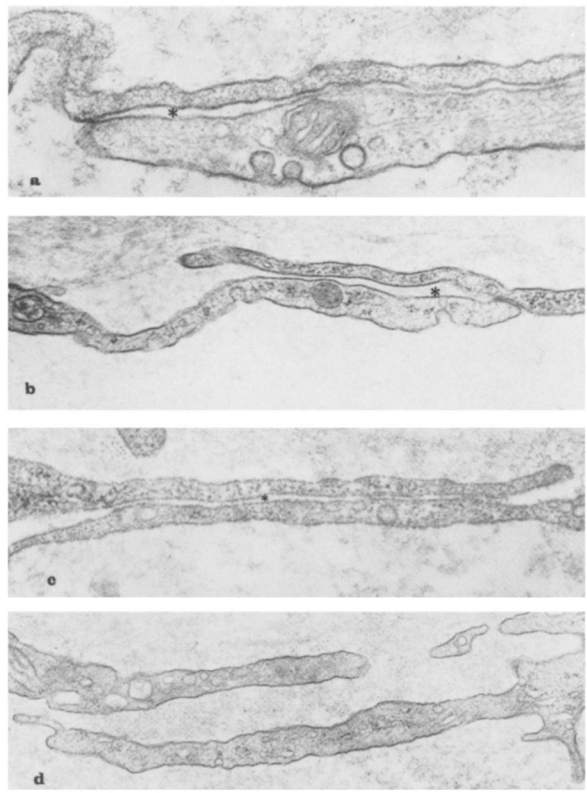

FIGURE 3. Electron micrographs depicting extensive overlapping of adjacent endothelial cell margins. The width of the intercellular cleft (*) is quite variable, ranging from areas of close apposition to regions where adjacent cells are widely separated to form patent junctions, as in (d). (Magnification: (a) × 76,500. (b) × 21,250. (c) × 33,150. (d) × 23,800.) (From Leak, L.V., *J. Cell Biol.*, 50, 300, 1971. With permission.)

anchoring filaments, 6 to 10 nm in diameter exist,[23] which are attached to the lymphatic capillary wall at one end and are embedded amidst the collagen fibers of the interstitium at the other end (Figure 4).[19] The anchoring filaments are spring-like structures which exert a pulling force on the capillary wall, thus preventing collapse of the capillaries, particularly during edema when the interstitial fluid pressure is elevated.

E. VESICLES

Invaginations of the plasma membrane are evident on both the luminal and abluminal surfaces of the endothelial cells and it is thought that these represent the formation or dissolution of cytoplasmic vesicles.[12] Studies using various tracer substances including ferritin, thorium dioxide[27] and horseradish peroxidase,[28,29] have shown that these vesicles do function in the transport of protein

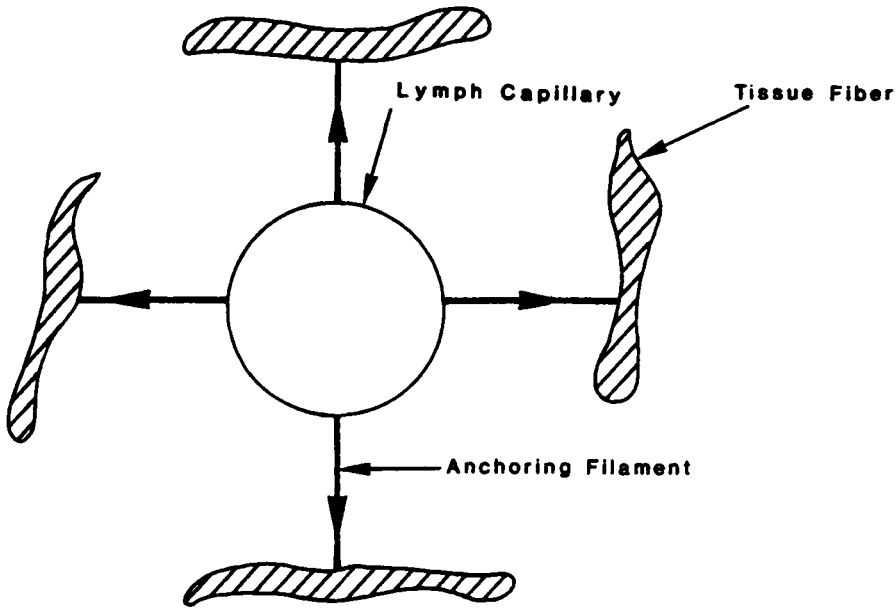

FIGURE 4. A diagrammatic representation of the anchoring filaments in relation to the tissue fiber matrix and the terminal lymphagion. (From Reddy, N. P., *Crit. Rev. Biomed. Eng.*, 14, 45, 1986. With permission.)

and macromolecules across the lymphatic endothelium. Occasionally, vesicles are of the coated type, but most are simple and smaller with diameters of approximately 75 nm.[20,24] Three possible destinations have been reported. Firstly, certain tracers may be transported to and stored in large vacuoles within the cell.[27] Alternatively, the vesicle may fuse with lysosomal vesicles causing digestion of the contents by lysosomal enzymes, the products of which are utilized by the cell or, finally, the vesicles may transport macromolecules across the entire cell and into the lumen of the vessel.[24,28]

F. LYMPH NODES

Lymph nodes are bean-shaped masses of tissue spread irregularly throughout the lymphatic system. The nodes range in size from 1 mm to 1 cm in diameter and there are approximately 500 to 1000 nodes present in the average adult human body. All lymph passes through at least one set of nodes and some passes through several sets before reaching the blood. The principal groups of nodes lie close to the blood vessels from which they often receive their names. The major

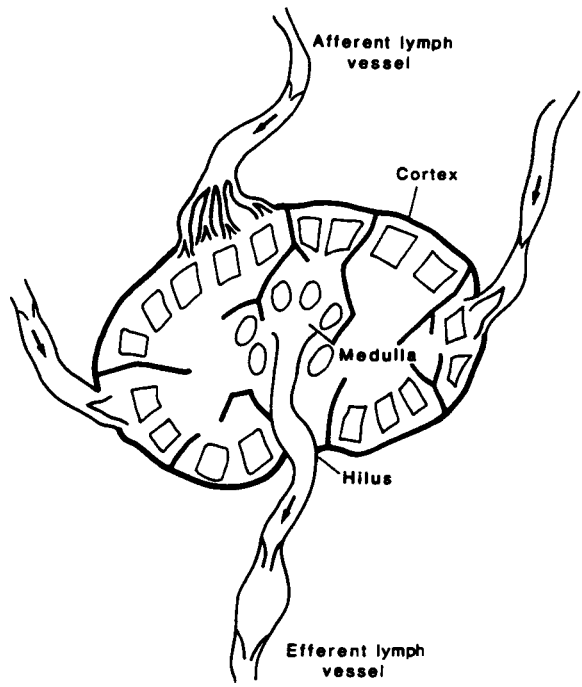

FIGURE 5. A diagrammatic representation of a lymph node. (From Reddy, N. P., *Crit. Rev. Biomed. Eng.*, 14, 45, 1986. With permission.)

function of lymph nodes is to filter the lymph. White blood cells are produced within the nodes. Foreign particles are filtered out by the parenchyma and toxins are neutralized by phagocytic cells in the node. In addition to the filtration process, exchange of various materials occurs between the lymph and the blood, thus altering the composition of the lymph. The mechanism of this exchange is poorly understood. The lymph node itself is enclosed in a capsule. Immediately beneath the capsule is a passageway called the subcapsular or cortical sinus which runs around the node. Below this sinus is a network of smaller sinuses referred to as the parenchyma of the node. Each incoming or afferent lymphatic divides upon the surface before entering the capsule (Figure 5).[19] It passes through the subcapsular sinus and through the cortical and medullary sinuses and exits the node at the hilum. Traditionally, it was believed that lymph nodes were not compartmentalized and that lymph from different afferent lymphatics was mixed and processed together. Saint-Marie et al.[30] have shown that physiological compartments exist within the node. Each compartment is assigned to a particular afferent lymphatic and the corresponding lymph is processed within that segment.

G. LYMPHATICOVENOUS ANASTOMOSES

Under normal conditions the major lymphaticovenous communication is at the junction of the thoracic duct and the subclavian and jugular veins. However, if a major lymphatic vessel becomes occluded the increased lymph pressure may force the opening of efferent lymphaticovenous junctions upstream to the occlusion point. The existence of such junctions has been established radiographically with lymphography.[31,32] Ligation of the thoracic duct has been shown to induce several communications between the thoracic duct and the azygos vein.[33,34] Lymphaticovenous junctions have been created artificially by microsurgery to treat clinical lymph edema resulting from obstruction of both upper and lower limbs.[35,36]

III. THE FORMATION OF LYMPH

The formation of lymph is influenced by the relative permeabilities of the blood capillary endothelium and the lymphatic capillary endothelium. Normal blood capillaries are permeable to proteins of molecular weight less than 40 kD which may leak out and accumulate in the interstitium. Persistent leakage and accumulation would eventually result in edema formation. To avoid this situation, the lymphatic system acts by removing the excess tissue fluids and plasma proteins, i.e., lymph, and returning it to the bloodstream. In this way the homeostasis of the interstitial environment is maintained. The permeability of the blood capillary endothelium is limited. Although freely permeable to water and molecules less than 40 kD molecular weight, it is only semipermeable to colloid of protein molecular size. Consequently, only relatively small amounts of the total plasma protein cross the blood capillary wall.[37–39]

A. PERMEABILITY OF THE LYMPHATIC CAPILLARY ENDOTHELIUM

The pathways taken, and the forces involved in the movement of interstitial fluid and proteins (lymph) across the lymphatic endothelium into the lumen of the capillaries, are the subject of ongoing research. Two major pathways have been suggested; the first is via intercellular junctions and the second via cytoplasmic vesicles. Most of the work to determine the relative contribution of the two routes to lymphatic capillary transport has been carried out using macromolecular tracers.

1. Intercellular Junctions

The role of the intercellular junction was investigated by Leak[27] following interstitial injection of ferritin, thorium dioxide, carbon, and latex spheres. The results obtained indicate that these tracer substances are transported into and out of lymph via the intercellular junctions rather than by vesicular transport (Figure 6).[27] These results have been supported by numerous other studies.[22,40–42]

The relative contribution of the two routes has also been examined following intravenous (I.V.) injection of tracers such as horseradish peroxidase (HRP)

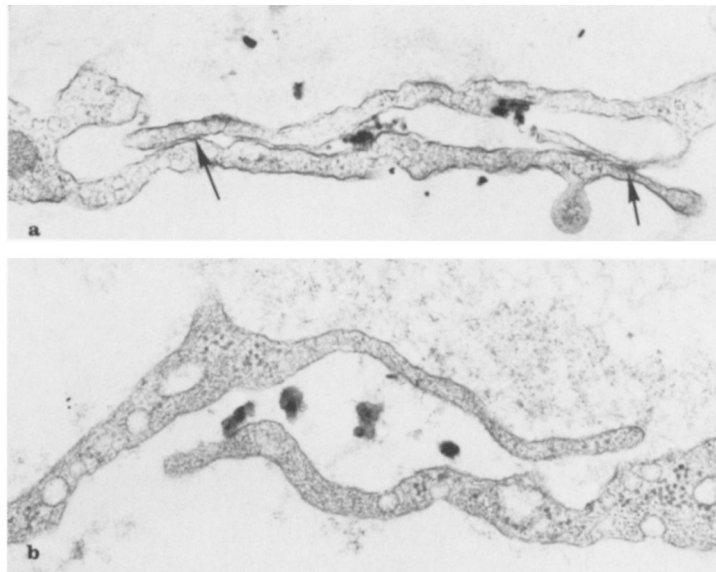

FIGURE 6. Electron micrographs illustrating the passage of colloidal carbon via the intercellular cleft. In (a) several areas of close apposition are found along the intercellular cleft (arrows), whereas in (b), the intercellular cleft forms an uninterrupted passageway. Specimens were injected with colloidal carbon 5 min before fixation. Lumina are at the bottom of the photographs. (Magnification: (a) × 24,480; (b) × 46,750.) (From Leak, L.V., *J. Cell. Biol.*, 50, 300, 1971. With permission.)

and ferritin.[24,25] These experiments, performed using renal lymphatics, are more physiologically relevant since the possibility of producing artificially high interstitial pressure is avoided by intravenous administration. The choice of HRP is particularly relevant since this molecule has a molecular weight of 40 kD and a diameter of 5 nm which is relatively close in size to that of plasma albumin (69 kD and a diameter of 7 nm). The results showed no significant difference in the proportions or widths of the intercellular junctions after administration of HRP or ferritin compared to the control. The tracer molecules entered renal lymphatics rapidly after I.V. injection and were transported in part via normal (20 to 30 nm) intercellular spaces between adjacent endothelial cells.[24]

Chylomicrons, which have a much larger diameter of approximately 200 nm, and are secreted in large numbers following a fatty meal, are transported into the intestinal lymphatics (lacteals) partly by the intercellular route (Figure 7).[28,43]

2. Vesicles

The studies described above of intercellular transport simultaneously assessed the role of vesicular transport, and the results imply that vesicular transport is also an important route for translymphatic transport and lymph

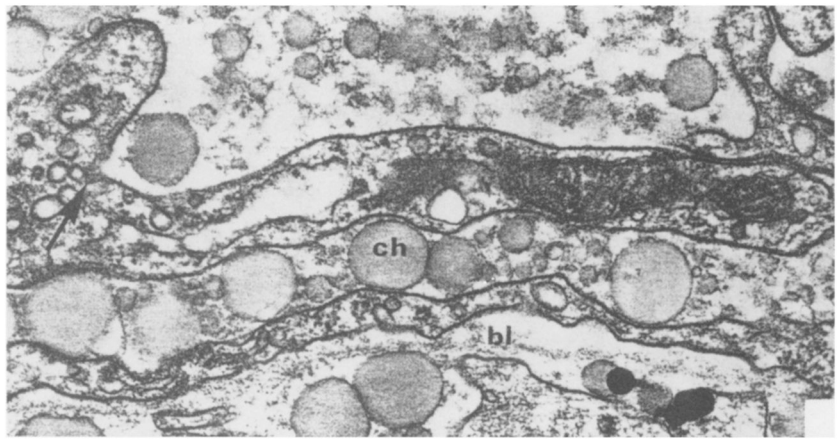

FIGURE 7. Lacteal endothelium of fat fed guinea pig showing numerous chylomicrons (ch) between overlapping endothelial processes and, at one point, possible extrusion of a chylomicron from a large pinocytotic vesicle (arrow). The basal lamina (bl) is also shown. (Magnification × 36,000.) (From Dobbins, W. O. and Rollins, E. L., *J. Ultrastruct. Res.*, 33, 29, 1970. With permission.)

formation.[20,24,27,28,44,45] Following I.V. injection of HRP, the volume density (i.e., the number) of small vesicles increased significantly and many of the vesicles contained the tracer.[24] The distribution of vesicles within the cytoplasm did not differ significantly from that of controls, thus indicating no major change in the mechanism of vesicular formation and movement. Yang et al.[24] therefore concluded that vesicles, if involved in translymphatic transport, may be stimulated by even a small increase in protein, in this study HRP, in the interstitium.

O'Morchoe et al.[29] developed an *in vitro* model using the canine renal hilar lymphatics to study the temperature dependence of protein transport across the lymphatic endothelium. The rationale behind this study was that if vesicular transport does occur then the rate of protein movement would be temperature dependent, in keeping with the known effect of temperature on vesicles, i.e., a decrease with decreasing temperature. In contrast, if the protein moves only by diffusion between adjacent cells then temperature would have little relative effect on the rate. The results demonstrated that the rate of protein transport was influenced by temperature, being significantly reduced by 49%, 31% and 29% when the temperature was lowered to 4°, 24° and 30° C, respectively, from an initial temperature of 37° C. When the temperature was raised to 40° C, the rate was significantly increased by 48%. No significant changes in intercellular junctional or vesicular parameters were found between the control lymphatics and those perfused at 24°, 30° and 40° C, thus indicating that the integrity of the tissue was maintained. At 4° C vesicular size decreased, the number of free cytoplasmic vesicles increased, but the number associated with the abluminal and luminal surfaces decreased. The authors concluded that the results obtained support the existence of a vesicular lymphatic transendothelial route.

FIGURE 8. Three dimensional diagram representing a portion of a lymphatic capillary, reconstructed from collated electron micrographs. The major passageway for transport of fluids and large molecules from the interstitium into the lymphatic lumen is by way of the intercellular cleft (long white arrows). The uptake of large molecules from both the connective tissue and luminal fronts may occur within vesicles (small arrows) which move towards the central cytoplasm. (From Leak, L. V., *J. Cell Biol.*, 50, 300, 1971. With permission.)

Dobbins and Rollins[28] demonstrated the role of vesicles in chylomicron transport in the intestinal lymphatics (Figure 7). Their results showed chylomicrons present within large pinocytotic vesicles opening to the abluminal side of the endothelia, others were completely enclosed within lacteal endothelia, and some were apparently "emptying" into the lacteal lumen. They concluded that the vesicular route is quantitatively more important than the intercellular route in the translymphatic transport of macromolecules including chylomicrons.

While results have been published in support of both intercellular and vesicular routes of transport across the lymphatic endothelium, the relative contribution of each individual route is not clear. It appears, however, that both routes function simultaneously (Figure 8)[27] and the importance of each may vary in different regions of the body and under different physiological conditions.

IV. LYMPH FLOW

Lymph flow or circulation can be broken down into two stages. The first stage involves lymph absorption and flow at the lymphatic capillaries via the intercellular or vesicular route. The second stage involves propulsion of the lymph along the larger contractile collecting lymphatic vessels.

A. LYMPHATIC CAPILLARIES

Different theories have been proposed to describe lymph filling or flow into the lymphatic capillaries. At the lymphatic capillary level there is extensive overlapping of adjacent endothelial cell walls. In addition, numerous anchoring filaments (af) are attached to the abluminal cell wall, except for the inner segments of the overlapped junction (Figure 9).[46] This arrangement creates a flap valve (trap door) effect which is free to move in response to pressure changes. As the interstitial pressure increases above that in the lymphatic lumen, this applies tension on the anchoring filaments, which in turn pull on the attached portion of the lymphatic capillary wall allowing the unsupported portion of the cell wall (i.e., the flap) to swing open into the lumen [Figure 9 (b) and (c)]. In contrast, when the lymphatic capillary luminal pressure exceeds that of the interstitium, the flap is closed against the overlapping cell wall.[46] Therefore, the pressure differentials between the interstitium and the lymphatic capillary lumen play a vital role in lymph formation and flow.

Casley-Smith[41,42,47] proposed an osmotic pull hypothesis to explain lymph flow through the lymphatic capillaries. This theory suggests that compression of the capillaries results in water expulsion out of the lymphatic capillaries into the interstitium through small pores impermeable to proteins. When the compressive force is released, the newly concentrated lymph exerts an osmotic force which draws fluid back into the capillaries via large pores. Considerable doubt has been cast on this theory due to recent studies which showed that protein concentration does not vary with vessel size[48] and that lymph protein concentration is the same as that in the tissue.[49]

An alternative possibility, namely the suction theory, has been proposed.[19,50,51] In this case it is argued that an increase in the interstitial pressure will in turn cause the intraluminal lymphatic pressure to rise. If the pressure in the lymphatic capillaries is greater than in the adjacent lymphangion (i.e., the segment between two adjacent valves), then lymph will flow from the capillaries into the lymphangion. Once the pressure within the larger vessel reaches its threshold, an active contraction is initiated in the lymphangion which propels the lymph forward into the adjoining lymphangion. During recovery from the contraction, the pressure within the lymphangion becomes negative and thus draws in further lymph from the capillaries. Consequently, in a type of chain reaction, the pressure within the lumen of the lymphatic capillaries becomes negative with respect to the tissue pressure, thus creating a suction within the lumen which starts the filling cycle over again.

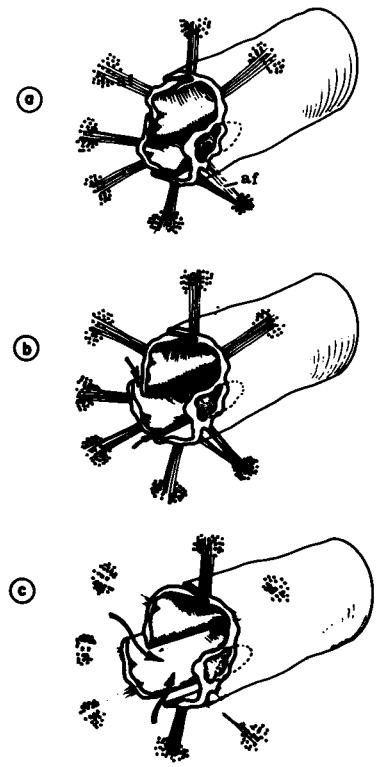

FIGURE 9. Diagrammatic representation of lymphatic capillary tissue interface. The anchoring filaments (af) facilitate expansion of the capillary lumen and regulate the direction of flow from the interstitial space into the lymphatic capillary. (From Leak, L.V., in *Handbuch der Allgemeinen Pathologie*, Messen, H., Ed., Springer-Verlag, Berlin, 1972, 149. With permission.)

B. COLLECTING LYMPHATIC VESSELS

In the collecting/larger lymphatic vessels, the walls become progressively thicker due to the appearance of connective tissue and smooth muscle. The thickest walls reveal a trilaminar structure similar to that of blood vessels. The intima consists of endothelium and the media contains smooth muscle cells laid down in one or two concentric layers enabling the vessels to contract and thus propel the lymph upstream. The adventitia is made up of loose connective tissue containing collagen, elastin, and fibroblasts. Small blood vessels and adrenergic nerves which service the needs of the lymphatic wall have been found within the adventitia.[12]

All the collecting lymphatics have a generous supply of valves, which occur at regular intervals, to prevent the backflow of lymph. However, the number and size of intravalvular segments have not been clearly established as they tend to be related to the vessel size.[52] Large variations in the structure of these valves

have been reported. Lauweryns[53] suggested a cone- or funnel-like design. Leak,[22] using scanning electron microscopy, found bicuspid valves along the lymphatics made up of two thin leaflets, the surfaces of which are lined with endothelial cells.[22,54] In the smaller lymphatic collecting vessels the valves consist only of endothelium. In the larger vessels, however, valves have several layers and contain elastic fibers. Tricuspid valves have also, on limited occasions, been reported.[19]

The propulsion of lymph along the collecting lymphatic vessels is governed by a number of different extrinsic and intrinsic factors. Extrinsic factors include muscle contraction, respiratory movements, and movements of the intestine. It has been shown that lymph flow increases during exercise and muscular activity.[55,56] Running has been shown to increase lymph flow from the leg by approximately 150 to 300% relative to the control.[57] Browse et al.[58] found that the pressure in the upper and lower ends of the dog thoracic duct was significantly influenced by respiration rhythm and Lee[59] showed that intestinal motility greatly influenced lymph flow from intestines.

Although these extrinsic factors have a role, it is now considered that the intrinsic rhythmic contractions of the valved lymphatic vessels are primarily responsible for the propulsion of the lymph toward the thoracic duct. The strength and frequency of these intrinsic contractions have been related to the rate of lymph flow.[60]

The intrinsic smooth muscle contractions of the lymphatic vessels are regulated by three major factors : (i) the transmural vessel distension, (ii) humoral mediators, and (iii) neural mediators.[19] The segment between two adjacent valves i.e., the lymphangion, contracts as a unit with the contraction being stimulated by radial distension. A contraction will only occur when the tension in the wall exceeds a certain threshold value, this value increasing with vessel size.[61] Each contraction lasts for a set period of time and is followed by a period of relaxation. The contractions begin in the lymphangions adjacent to the lymphatic capillaries and spread progressively through each lymphangion towards the thoracic duct. The contractile cycle of the lymphatic smooth muscle has not been extensively examined. However, it seems that there are two separate phases: (i) the diastolic phase, during which the intervalvular segment is filling, and (ii) the systolic phase, during which the contraction develops.[62]

Zweifach and Prather[63] performed a detailed study of collecting lymphatics of the mesentry of the cat and the omentum of the rabbit. The results showed that contraction begins in the smaller branches and spreads progressively to the larger vessels, this process taking 4 to 5 seconds. Between 10 and 18 contraction-relaxation cycles occurred per minute with each contraction cycle lasting on average 3 to 4 seconds. The amplitude of pulsations ranged from 2 to 4 cm H_2O in the larger lymphatics and the intraluminal pressure increased gradually with vessel size.

A range of humoral mediators including histamine, epinephrine, norepinephrine, 5-hydroxytryptamine (5-HT), prostaglandins, dopamine, acetylcho-

line, phentalomine, isoproterenol, ATP, and ADP have been shown to affect lymphatic smooth muscle contractions.[64] Anesthetics, including halothane[65] and pentobarbitone,[56] have been shown to inhibit lymph flow.

Isolated segments of lymphatic tissue have been found to respond to electrical stimulation.[66–68] These results suggest an adrenergic response. Stimulation of intramural nerves in the lymphatics has been suggested as an alternative method for the control of lymph propulsion.[69,70]

The large variations in lymph flow rates which have been reported in the literature may be due to the many factors which affect lymph flow. Lymph flow rates are enhanced during fluid and food absorption, with up to tenfold increases in lymph flow being reported after food.[1] Lymph flow rates vary in different sites throughout the body and inter-species variation is also evident.[1] The thoracic duct lymph flow in mammals varies from about 1 to 2 ml/kg/hr in man, giving an average of about 2 liters per day for an individual of 70 kg body weight, to 3 to 5 ml/kg/hr in ruminants. In rats, which are frequently used in experimental lymphatic studies, the following average ranges have been reported for animals weighing approximately 200 gm: thoracic duct 12 to 25 ml/day, intestinal vessels 21 to 22 ml/day, and hepatic vessels 2 to 5 ml/day. Various mathematical models have been developed to describe lymph formation and flow.[19,50,51,71,72]

V. COMPOSITION OF LYMPH

In general the chemical composition of lymph is very similar to that of plasma and many components differ only in concentration. The concentration of various components in the lymph from different regions in the body varies and there is also inter-species variation. A very comprehensive review of these factors is given by Yoffey and Courtice.[1] The major components of the lymph are as follows.

A. PROTEINS

Lymph contains all the protein fractions of plasma but at a lower concentration. Thoracic duct lymph in man was found to have a protein concentration of about 66% that of serum.[73] The lymph to blood plasma concentration ratios (L/P) for albumin and total globulin were approximately 0.75 and 0.5, respectively.

B. ENZYMES

Many of the enzymes present in plasma are also found in the lymph, although generally at a lower concentration. However, in lymph draining a tissue in which the enzyme is produced, the concentration may be higher than in plasma. The concentration of alkaline phosphatase in the intestinal lymph is higher than that in plasma.[1] Enzymes which have been measured in the human thoracic duct lymph include alkaline phosphatase, acid phosphatase, lactic dehydrogenase, glutamic-oxaloacetic transaminase, glutamic-pyruvric transaminase, and aldolase.[74]

C. LIPIDS

The lipids found in lymph are similar to those in plasma. The concentrations in lymph are lower except in the case of intestinal lymph. These lipids, including triglycerides, phospholipids, cholesterol esters, and free cholesterol, are present in lymph as lipoproteins.[75] When mesenteric lymph collected after fat ingestion was examined by sequential gradient ultracentrifugation, lipoproteins including chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were detected.[76] These lipoproteins differ in size and in lipid and apoprotein composition.

Chylomicrons are the largest (75 to 600 nm) lipid carrying particles in the lymph.[77] Production of chylomicrons is phasic and dependent upon co-administration of absorbable lipid. However, it is generally assumed that the concentration of chylomicron lipid within intestinal lymph is approximately 1 to 2%.[78–80] Chylomicrons have the following approximate composition: 86 to 92% triglyceride, 0.8 to 1.4% cholesterol esters, 0.8 to 1.6% free cholesterol, 6 to 8% phospholipids, and 1 to 1.5% protein.[81] The core of the chylomicron contains most of the triglycerides and cholesterol esters along with about 30% free cholesterol. The surface of the chylomicron is composed of a monolayer of phospholipids, apoproteins, free cholesterol, and a small amount of saturated triglycerides.[82]

D. ELECTROLYTES

The electrolyte content of lymph is not significantly different from that of plasma.[1] Generally, the concentrations of cations including sodium, potassium, calcium, and magnesium are slightly lower, while the anions, chloride, and bicarbonate are higher in lymph than in plasma.

E. NONELECTROLYTES

The level of nonelectrolytes, including nonprotein nitrogen, urea, amino acids, and creatinine, are all approximately the same in both plasma and lymph.[1] An exception occurs in renal lymph where the level of creatinine is lower than that in plasma. Glucose levels in both thoracic and intestinal lymph are similar to plasma, although higher glucose levels have been found in hepatic lymph.[83]

F. IRON AND TRANSFERRIN

Iron remains bound to transferrin during its passage from blood to lymph.[84] Werner[74] reported average values for transferrin-bound iron of 71 μg/100 ml in thoracic duct lymph and 90 μg/100 ml in plasma.

G. COAGULATION FACTORS

Lymph from all regions of the body clots but generally less readily than plasma. The concentrations of fibrinogen and prothrombin in lymph are less than in plasma and vary considerably in different parts of the body.[1]

VI. COMPARATIVE MORPHOLOGY OF LYMPHATICS AND BLOOD VESSELS

A. MORPHOLOGY

A study of the morphology of both lymphatic and blood capillaries is relevant as it may have a significant effect on the permeability of these vessels. Any alteration in permeability will in turn influence microvascular exchange, i.e., transport and exchange of fluid, small solutes, and macromolecules between the capillaries, the interstitium, and the lymphatics.

The detailed morphology of the lymphatic capillaries has been described earlier. In summary, it consists of a single layer of nonfenestrated endothelial cells which lack a continuous basal lamina. In contrast, three different types of blood capillaries exist within the body containing continuous, fenestrated or discontinuous endothelia.[85] The structural variation of these capillaries presumably accounts for the regional differences found in capillary permeability.

1. Continuous Capillary Beds

Capillaries with continuous endothelia and an uninterrupted basement membrane are the most widely distributed in mammalian tissues. Tissues possessing continuous capillaries include subcutaneous tissue, lung, skeletal muscle, myocardium, nervous tissue, and adipose tissue. The possible transport pathways across these cells, as shown in Figure 10, include pinocytotic vesicles, intercellular junctions, and transendothelial channels.[86]

2. Fenestrated Capillary Beds

Capillaries with fenestrated endothelia have a continuous basement membrane and are usually found in organs with high rates of fluid exchange, e.g., kidney, small intestine, and salivary glands. The capillaries in the kidney have a unique structure. Most of the work published on the permeability of fenestrated capillaries deals with the gastrointestinal capillaries. The possible transport pathways across these cells include diaphragmed fenestrae, open fenestrae, intercellular junctions, pinocytotic vesicles, and basement membrane (Figure 10). Traditionally, the fenestrated capillaries have been thought of as being leaky. However, recent studies on the permeability of intestinal capillaries to endogenous macromolecules have shown that these capillaries selectively restrict macromolecules to a greater degree than the continuous type found in other organs.[87]

3. Discontinuous Capillary Beds

Capillaries with discontinuous endothelia have no basement membrane and are found in liver, spleen, and bone marrow. Transport pathways across the cells include pinocytotic vesicles and intercellular gaps, with diameters ranging between 100 nm and 1 μm (Figure 10).

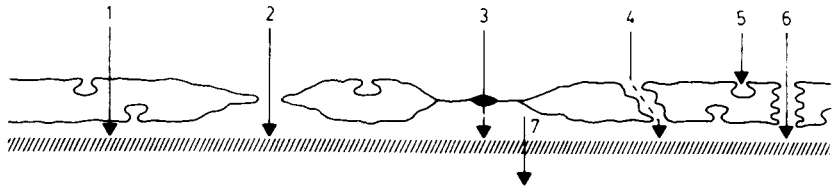

FIGURE 10. Diagrammatic representation of transport pathways in capillaries. Transport pathways: (1) cell membrane; (2) open fenestrae; (3) diaphragmed fenestrae; (4) intercellular junction; (5) pinocytotic vesicles; (6) transendothelial channels; (7) basement membrane. (From Granger, D. N., and Barrowman, J. A., *Gastroenterology*, 84, 846, 1983. With permission.)

VII. ROLE OF THE LYMPHATICS IN EXCHANGE IN NORMAL TISSUE

For a full understanding of the role of the lymphatics in fluid and solute exchange in normal tissues, the role of the corresponding blood capillaries must also be considered. The permeability of the blood capillaries influences the passage of fluid and solutes out of the blood vessels and into the interstitium which may eventually end up in the lymph. Similarly, the permeability of the blood capillaries will influence the return of material from the interstitium away from, or in direct competition to, the lymph. Consequently, the two systems complement each other to maintain fluid balance.

A. INTERACTION OF CAPILLARY, INTERSTITIAL, AND LYMPHATIC FORCES

The rate and direction of fluid movement across capillaries is governed by the hydrostatic and oncotic pressure gradients between the blood and interstitium.[88] This relationship was described by Starling using the following equation:

$$J_{vc} = K_{f,c}[(P_c - P_t) - S_d(\pi_c - \pi_t)] \quad (1)$$

where J_{vc} is the net capillary filtration (or absorption) rate, $K_{f,c}$ is the capillary hydraulic conductance, P_c is the capillary hydrostatic pressure, P_t is the interstitial fluid pressure, S_d is the osmotic reflection coefficent, π_c is the oncotic pressure in the capillary, and π_t is the interstitial oncotic pressure.

The rate of lymph flow from a tissue is assumed to give an estimate of net capillary filtration rate under isovolumetric or isogravimetric conditions (i.e., when the tissue is neither gaining nor losing weight). Movement of fluid out of the blood capillaries into the interstitium will increase the interstitial to lymphatic capillary hydrostatic pressure gradient and, hence, affect lymphatic filling and lymphatic flow.[89]

Capillary filtration coefficients ($K_{f,c}$) are a measure of the hydraulic conductance of a capillary bed and are influenced by the size and number of pores in each

capillary in addition to the number of perfused capillaries.[90] It is generally accepted that two populations of "pores" exist within blood capillary membranes, the "small pores" (4 to 5 nm radius) which are more plentiful and the "large pores" (approximately 25 nm radius), which are mainly associated with the transport of macromolecules.

Capillary pressure (P_c) is influenced mainly by arterial and venous pressures. Interstitial fluid pressure (P_t) is principally determined by interstitial fluid volume and, because the blood capillaries are permeable to plasma proteins, only part of the oncotic pressure generated by plasma proteins is exerted across the capillary wall. The osmotic reflection coefficient (S_d) describes that fraction of the total oncotic pressure which is generated across the blood capillary wall. Lymph protein data have been used to determine values of osmotic reflection coefficients. A comparison of lymph to plasma solute concentration ratios (L/P) for a range of molecular radii provides an estimate of the permeability of the capillary wall and facilitates the calculation of S_d ($S_d = 1 - L/P$).

If the lymph is similar in composition to interstitial fluid then the transcapillary oncotic pressure gradient ($\pi_c - \pi_t$) can be estimated from lymph and plasma using either an oncometer or equations that relate protein concentration to oncotic pressure.

An increase in venous pressure results in an increase in capillary pressure which in turn causes an instantaneous rise in net filtration pressure. This brings about an enhanced fluid movement from the vascular system into the interstitium. As the interstitial fluid volume expands, P_t rises and π_t falls. These readjustments in interstitial forces reduce the net filtration pressure, thereby opposing excess capillary filtration. The elevated P_t drives interstitial fluid into the lymphatics and a new steady state is reached, with a slightly increased interstitial volume and a markedly elevated lymph flow.

B. SMALL MOLECULE TRANSPORT

Small molecules (molecular weight < 2,000), including water, are predominantly transported via the blood vessels.[37,91] The relative proportions of water leaving the interstitium via the lymphatic and blood capillaries tend to vary, e.g., in the event of threatening edema or in the intestine during fat absorption.

C. MACROMOLECULE TRANSPORT

The lymphatic system transports 50% of the total plasma protein pool back to the blood every 24 hours.[1] A continuous slow leak of these plasma proteins from blood vessels into the interstitium occurs via convection, diffusion, and vesicular exchange, partly in response to the concentration gradient from blood to tissue. These proteins, consequently, enter the lymph, thus avoiding protein accumulation in the tissues.

Granger et al.[92] studied the movement of plasma proteins across the intestinal capillaries in the absorbing small intestine. During absorption the movement of fluid is from the interstitium to the blood. In this situation,

convection and diffusion are moving proteins in opposite directions across the capillary wall. Consequently, during absorption significant movement of plasma proteins into and out of the mucosal interstitium occurs. The reason for the large interstitial exchange of plasma proteins is unclear. It has been suggested, however, that it may provide a mechanism whereby absorbed nutrients are removed from the interstitium bound to plasma proteins. The proposed mechanism is that unbound proteins diffuse out of the circulation, bind to the absorbed nutrient, and are carried back into circulation by the convective movement of the absorbed fluid. Such a mechanism may be important for the absorption of medium and long chain fatty acids that are not incorporated into chylomicrons but enter the circulation in significant amounts bound to albumin.[93]

D. QUANTITATIVE RELATIONSHIPS BETWEEN LYMPHATICS AND BLOOD VESSELS

Deak and Csaky[94] studied factors regulating the exchange of nutrients and drugs between lymph and blood in the small intestine. They concluded that molecular size significantly affects the relative distribution. The classic example of the effect of molecular size and molecular sieving on lymphatic transport is the chylomicron. These particles are too large to enter directly the fenestrated blood capillaries of the mucosa and, consequently, are transported solely via the lymph.

This molecular sieving mechanism may be potentially exploited for macromolecular polypeptide drug delivery as discussed in Chapter 7. Muranishi et al.[95] evaluated the absorption route, i.e., lymph vs. blood, of macromolecules following rectal administration. They showed that small amounts of the administered dose of macromolecules could pass the epithelium of the rectum with the help of enhancers. Once past the epithelium they do not readily enter the blood vessels but, instead, are transported principally via the lymphatics. The molecular weight range of the macromolecule is vital to maximize this effect. A macromolecule smaller than 10 kD may be transported into both fluids at about the same concentrations, whereas a protein with a molecular weight of approximately 20 kD may primarily enter the lymphatics.

The relative amount of absorbed fluid that is removed from the interstitium by the lymphatics and the capillaries has been investigated.[96] In the past, estimates of the proportion of absorbed water leaving the intestine via the lymphatics ranged from 1 to 85%. Recent studies show that the rate of fluid absorption has a major effect on the absorbed volume which is removed by the lymphatics of the small intestine. At normal and high fluid absorption rates, the relative contributions of each system remain relatively constant at 80 to 85% for capillary removal and 15 to 20% for lymphatic removal. In contrast, lymph flow in the colon is unaffected by fluid absorption, primarily due to the scarcity of lymphatics in the colonic mucosa.[97]

Perfusion of the intestinal lumen with a glucose electrolyte solution resulted in glucose absorption induced changes in capillary and interstitial forces which

favored the removal of 80% of absorbed fluid by blood capillaries and 20% by lymphatics.[88] During lipid absorption, however, Granger et al.[98] showed that microvascular and lymphatic fluid dynamics are quantitatively different than those produced by glucose absorption. During lipid absorption 35% of the absorbed fluid is removed from the mucosal interstitium by lymphatics, while blood capillaries remove the remaining 65%. These differences are explained by lipid absorption induced increases in blood flow (e.g., long chain fatty acids almost double intestinal blood flow),[99] and increases in microvascular permeability. The increased lymph formation during lipid absorption is physiologically advantageous in that it allows for an enhanced convective transport for chylomicrons from the mucosal interstitium to the systemic circulation.[100]

The molecular sieving characteristic of the blood/lymph barrier has also been examined.[94] Following intravenous administration of two different molecular weight dextrans, a significant decrease in the lymph/plasma concentration ratio was seen for the higher molecular weight dextran.

The uptake of proteins appearing in the tissue *de novo* was investigated by using increased metabolism and injurious stimuli to induce the release of enzymes (20 to 100 kD) from various regions.[101,102] Results showed that significant amounts of these proteins passed into both blood and lymph. In nonfenestrated regions the amount of enzymes in the blood tended to be only slightly greater, when corrected for blood flow. In contrast, in the fenestrated regions (e.g., intestine and liver), the amounts passing into the blood were noticeably higher compared to the lymph.

VIII. LYMPHATICS IN SPECIFIC ORGAN TISSUES

The anatomy and physiology of the lymphatic system varies in different regions of the body.[1] A limited number of organ tissues which have potential as sites for lymphatic drug delivery will be discussed. Targeting drugs into the lymph has certain advantages, which arise mainly as a result of the unique anatomy and physiology of the lymphatic system. These include avoidance of first pass metabolism, direct delivery of cytotoxic drugs in the treatment of lymphatic cancer, and the possibility of regulating the rate of drug delivery.[103]

A. SMALL AND LARGE INTESTINES

1. Anatomy of the Lymphatic System

The lymphatics of the small intestine are characterised by the presence of a centrally located vessel, called a lacteal, in each villi (Figure 11).[97] The lacteals are approximately 20 μm in diameter and are located about 50 μm from the epithelial cells. The lacteals join a plexus of lymphatic capillaries in the glandular layer of the mucosa and then pass down to a submucosal network of collecting lymphatics. Numerous anastomoses exist between the lymphatics of the mucosa and the submucosa. The superior mesenteric collecting lymphatic vessel drains the small intestine and the ascending and transverse colon, while the inferior

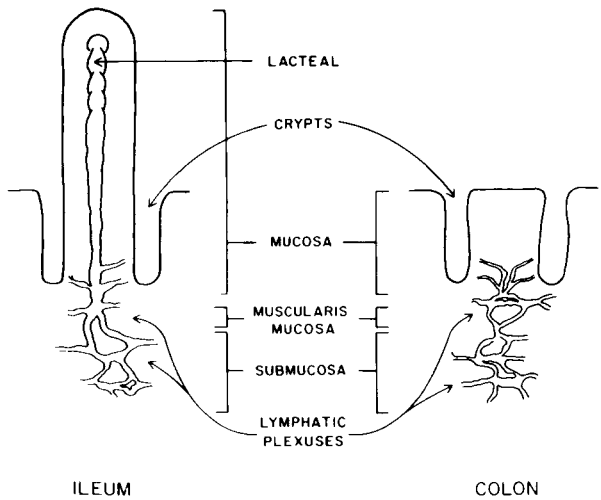

FIGURE 11. Schematic representation of the mucosal-submucosal lymphatic microcirculation of the canine ileum and colon. (From Kvietys, P. R., Wilborn, W. H., and Granger, D. N., *Gastroenterology*, 81, 1080, 1981. With permission.)

mesenteric vessel drains the descending and sigmoid colon. The mesenteric lymph drains into the cisterna chyli and is returned to the general circulation via the thoracic duct, thus avoiding the liver and potential first pass metabolism.[96,104,105] Cannulation of the mesenteric lymphatic vessel is essential if a true estimate of intestinal lymphatic transport, following oral administration, is required. Collection of thoracic duct lymph will result in an overestimation of intestinal transport, due to contamination of thoracic lymph with lymph from other tissues (see Chapter 3).[106]

In the large intestine the lymphatic vessels are fewer in number and smaller in size (Figure 11). They originate deep in the mucosa near the bases of the glands and are 300 to 400 μm from the surface epithelium. As in the small intestine, numerous anastomoses exist between the lymphatics in the mucosa, the muscularis mucosa, and the submucosa.

It has been shown that the protein concentration of the fluid within the lacteals is similar to that in the collecting lymphatics.[107] This indicates minimum contributions from the submucosal and muscle regions. In contrast, the scarcity and smaller size of the colonic lymphatics imply that lymph draining the large intestine is derived mainly from the muscle layer of the wall.

2. Physiology of the Lymphatic System

Intestinal lymph flow rates vary between species and are influenced by fluid and food intake. A large difference exists between intestinal lymph and blood flow rates. The relative flow of portal blood to intestinal lymph is approximately 500:1.

The intestinal lymphatic system plays a vital role in removing fluid and proteins which escape from the blood circulation, and in transporting absorbed fluid and some nutrients from the lamina propria. Intestinal microvascular exchange has been extensively studied and reviewed by Granger and co-workers.[85,88,108] The rate of lymph formation in the small intestine is influenced mainly by the rate of fluid filtration across the capillaries and the rate of fluid absorption by the mucosal membrane, both of which will affect the interstitial hydrostatic and oncotic pressure.

Mortillaro and Taylor[109] have shown, using the cat ileum, that a sigmoidal relationship exists between steady state lymph flow and interstitial fluid pressure (P_t). Increasing P_t within the range of 0.10 to 1.80 mm Hg did not significantly alter lymph flow. However, when P_t was increased to 3 to 5 mm Hg, lymph flow increased dramatically. When P_t was greater than 5 mm Hg, lymph flow remained relatively constant. This sigmoidal relationship between P_t and lymph flow is expected to occur whether P_t is increased due to enhanced capillary filtration or transmucosal fluid absorption. Filling of the lacteals is controlled by a combination of intrinsic and extrinsic factors. Lacteals in man do not exhibit rhythmic contractions.[104]

Womack et al.[110] have shown a highly significant correlation between villous contraction frequency and lymph flow. Villous contraction may be stimulated by amino acids, lipids, including a mixture of taurocholate and oleic acid, and also by fluid absorption.[110,111]

3. Chylomicron Transport

As a result of lipid digestion and absorption, chylomicrons are synthesized within the enterocytes. The chylomicrons are excreted from the enterocytes by exocytosis and as they are too large to enter the blood capillaries, they are taken up exclusively via the lymphatics.[112] After leaving the enterocyte, the chylomicron must travel across approximately 50 μm of interstitium before arriving at the central lacteal where the particles enter through both interendothelial cell junctions and vesicles.[28,112,113] Removal of chylomicrons from the mucosal interstitium is one of the major functions of intestinal lymphatics.

Chylomicrons appear to traverse the interstitium without difficulty, in spite of the resistance offered by the interstitial gel to such large particles.[114] One possible explanation involves nonendothelialized channels which have been identified in certain tissues and are called "prelymphatics".[115,116] However, there is no morphological data to support chylomicron transport via these channels.

Net volume absorption results in an increase in intestinal interstitial volume causing interstitial hydration, an increase in lymph flow and a decrease in the degree of albumin exclusion in the interstitial matrix.[117] Tso et al.[100] examined the effects of interstitial hydration and lymph flow on chylomicron transport rate. The results show that lymph flow has a significant effect on intestinal chylomicron transport. As lymph flow increased, the chylomicron appearance time (i.e., the time between the placement of radioactive fatty acid into the intestinal lumen

and the appearance of radioactive lipid in the central lacteal) was reduced. When lymph flow exceeded 40 μl/min, the chylomicron appearance time reached a minimum value of approximately 14 min. The authors suggested that this time may represent the time required for assembly of absorbed lipid, formation of chylomicrons, and their subsequent discharge into the lymphatics.

Tso et al.,[118] however, noted that lymph flow both determines the rate of convective movement of chylomicrons and reflects the degree of interstitial hydration. Consequently, a further study was performed to determine which factor was more important for the inverse relation between the chylomicron appearance time and lymph flow. The authors concluded that the reduced chylomicron appearance time produced by expansion of the mucosal interstitium resulted from a diminished resistance of the interstitial matrix to chylomicron movement, rather than a decreased transit time due to an enhanced convective flux of chylomicrons.

Intestinal lymphatic transport of lipophilic compounds has been shown to occur in association with chylomicrons.[119–121] As described in Chapter 4, co-administration of lipid vehicles, which stimulate chylomicron production, have been used to enhance lymphatic transport following oral administration.[5,7,122,123] The loading or concentration of drugs per chylomicron is influenced by both the partition coefficient and the lipid solubility.[78] Charman et al.[121] demonstrated that the hourly intestinal lymphatic transport of DDT was determined mainly by chylomicron flux and not lymph flow.

4. Fluid Secretion

Under certain conditions (e.g., cholera) the small intestine actively secretes protein free fluid into the intestinal lumen. Theoretically, this fluid secretion should result in a reduced mucosal interstitial fluid volume and pressure with a subsequent decrease in lymph flow. Experimental results which concur with this theory have been published.[124] Colonic lymph flow, however, is not altered by stimulation of active secretory processes.

If an imbalance in the forces governing transcapillary exchange occurs which causes movement of fluid out of the vascular system and into the interstitium, this will result in an increase in interstitial fluid pressure and an increase in lymph flow. These responses oppose filtration and are referred to as "edema safety factors".[86]

B. SKIN AND SUBCUTANEOUS TISSUES

1. Anatomy of the Lymphatic System

The lymphatics of the skin consist of a superficial lymphatic plexus in the dermis, which extends to the outer two-thirds of this structure, and another plexus lying in a deeper part of the dermis adjacent to the subcutaneous tissue. The superficial plexus is without valves; however, valves are present in the deeper plexus occupying the inner dermis and the subcutaneous tissue. No lymphatic vessels have been found in the epidermis. This basic structure is found

in skin throughout the body. However, certain areas such as the fingers, palm of the hand, sole of the foot, and the scrotum appear to have a richer supply of lymphatics. The shape of the lymphatics are uniform where the skin is thicker and rigid, whereas the shapes are more variable in regions where the skin is thinner. The exact reason for these differences is unknown, but it has been suggested that it may be due simply to variations in external pressures applied to the lymphatic walls.[125] The lymphatics found in the interior of the dermis join anatomically with the subcutaneous lymphatics and form Jossifow's cutaneous lymphatic plexuses.[1,91,125]

2. Physiology of the Lymphatic System

Studies of the lymphatic drainage of the skin have been performed mainly using animal paw/leg preparations. In these systems, the popliteal lymphatics, which drain the skin and subcutaneous tissue and are associated with the popliteal node, are cannulated. Lymph flow from this area is approximately 1 ml/hr/100 g, but may increase 12- to 13-fold when venous pressure is raised.[126] Lymph flow through the lymphatics of the skin may be influenced by contraction of the surrounding tissues, pulsation of neighboring blood vessels, and respiration. An intrinsic ability to contract is seen only in the larger, more muscular lymphatics.[60]

The paw preparation is the only tissue where the general assumption that the protein composition in lymph equals that of the interstitium has been experimentally proven.[127] The blood capillaries in the skin are of the continuous type, hence permeability is low and only small amounts of plasma proteins leak into the interstitium. At normal lymph flow the protein content of paw lymph is approximately 2 g % compared to 6.5 g % for plasma, and it decreases further when venous pressure is raised.[126] Garlick and Renkin[128] studied the transport of large molecules from plasma to interstitial fluid and lymph. Test molecules ranging from 8 to 500 kD were administered intravenously and lymph was collected via the popliteal lymphatics in the skin. They concluded that macromolecular transport across the capillaries could be explained by small pores, 4 nm in radius, and a few larger pores (radius 80 nm), or pinocytotic vesicles to carry the larger molecules. The interaction of capillary, interstitial, and lymphatic forces in the canine hindpaw, under normal conditions, and the role of lymph flow in the prevention of edema has been studied by Chen et al.[126]

3. Drug Delivery

Supersaxo et al.[129] studied the effect of different parenteral administration routes [intradermal (I.D.), subcutaneous (S.C.), and intravenous (I.V.)], infusion rates, and albumin content of the drug vehicle on the cumulative recovery of recombinant human interferon alpha-2a (rIFN α-2a) in lymph, and on its concentration in the blood and lymph of sheep. Blood samples were taken from the jugular vein and lymph was collected via cannulation of the efferent vessel of the popliteal lymph node. The depth of injection was 2.25 mm for the S.C.

administration and 1.25 mm for the I.D. administration. The results show that following I.D. or S.C. administration into the cannulated leg approximately 59.2% of the administered dose of the rIFN α-2a was recovered in the popliteal lymph. On the basis of this result, the authors suggested that following I.D. or S.C. administration, rIFN α-2a (19 kD) was transported mainly by the lymphatics draining the site of application. The large size of the molecule, together with the structural or permeability differences between blood and lymph, were suggested as the reasons for the selective delivery into the lymphatics. The cumulative recovery of rIFN α-2a in the lymph following S.C. or I.D. administration was unaffected by either infusion rate or by co-administration of albumin. Very low levels, approximately 0.002% of the administered dose, were detected in the popliteal lymph following I.V. administration. It should be noted, therefore, that significant delivery of rIFN α-2a into the lymph was only achieved when administered locally. In contrast to the results of Supersaxo et al. in sheep, previous workers have reported very low values, approximately 0.04% of the administered dose of interferon recovered in thoracic duct lymph of rabbits following S.C. administration.[130,131]

A further study was performed on the effect of molecular weight on the lymphatic transport of water-soluble compounds following S.C. administration by cannulation of the efferent duct of the popliteal lymph node.[132] The results showed that in the molecular weight range used (i.e., 246 to 19,000), there was a linear relationship between the molecular weight and the proportion of the dose transported lymphatically. An increase in molecular weight resulted in an increased lymphatic absorption. The authors concluded that molecules with molecular weight greater than 16 kD, which are administered S.C., are transported mainly by the lymphatics which drain the application site. The combined data from these studies indicate that the S.C. route of administration may be an effective route of delivery for protein drugs (e.g., interferons and interleukins) whose targets are lymphoid cells.

C. SKELETAL MUSCLE

1. Anatomy of the Lymphatic System

The lymphatic capillaries are confined to the fascial planes around the muscle bundles and none have been detected in the endomysium between muscle fibers.[1,133] Studies on the leg muscles in mammals have shown two main systems of lymphatic drainage, the superficial and the deep.[134] The superficial lymphatics are located in, or close to, the subcutaneous tissue and enter either the popliteal or the inguinal lymph nodes. Due to the close proximity of skin lymphatics, it is often very difficult to obtain lymph which is uncontaminated by sources outside muscle. The deep system of lymphatics is located near the deep blood vessels of the muscle in the lower limb.

Jacobson and Kjellmer[135] cannulated the femoral lymphatics in cats and dogs and were able to exclude most of the lymph from the skin by placing a ligature around the paw. With passive movement of the muscle, ligation of the

paw decreased lymph flow from 25 to 5 µl/min per limb. These results indicate that femoral lymph predominantly originates from skin.

Bach and Lewis[136] collected muscle lymph from the hindlimb of rabbits by cannulating the femoral lymphatics distal to the inguinal node and ligating the efferent lymphatic from the popliteal node. In this way, contamination from the superficial lymphatics was reduced or eliminated and it was assumed that the lymph collected was in fact from the deep muscle lymphatics. On comparison with lymph flow from a prepopliteal superficial lymphatic which drains the skin, and having corrected for tissue mass, the authors concluded that lymph flow from the skin was much higher than that from muscle.

Muscle lymph has a total protein concentration twice as high as skin lymph under normal steady-state conditions.[137] The blood capillaries in both muscle and skin are "continuous". Permeability studies using a range of macromolecules have indicated that capillary permeability is similar for both tissues.[85] It has been suggested that the higher lymph protein concentration may be related to a lower net fluid filtration across muscle capillaries.[138]

IX. EFFECT OF ADMINISTRATION ROUTE ON LYMPHATIC DELIVERY

While attempting to target or optimize transport of drugs to the lymphatic system (e.g., for the treatment of cancer metastasis) in addition to evaluation of formulation variables, consideration should be given to the route of administration because this factor has been shown to affect lymphatic delivery.[129,130,132,139]

In order to identify the administration route giving the most selective uptake of human tumor necrosis factor (rHu-TNF) into lymph, Kojima et al.[139] examined the plasma and lymph levels of rHu-TNF after systemic and various local administrations. The results showed that intramuscular (I.M.) and subcutaneous (S.C) administrations gave low levels of rHu-TNF in both plasma and lymph. In contrast, following intra-stomach wall (S.W.), intra-gut wall (G.W.), and intraperitoneal administration (I.P.), the ratios of rHu-TNF recovered from the thoracic duct to the cumulative amount estimated from the rHu-TNF concentration in plasma were about 25, 32 and 8 times higher, respectively, than in the case of I.V. administration. The authors suggest, therefore, that routes of local administration, such as S.W., G.W., and I.P., may be highly effective in the treatment of lymphatic metastasis of cancer.

X. CONCLUSION

The anatomy and physiology of the lymph has been described. The relevant aspects which may affect the success of the lymphatic route as an alternative pathway for drug delivery have been elucidated. Succeeding chapters will present in detail the current status and future prospects for the lymphatic transport of drugs.

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Chapter 2

INTESTINAL PROCESSING OF DIETARY LIPIDS

Charles M. Mansbach, II

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I. INTRODUCTION

This chapter will outline the major features of lipid absorption and highlight the multiple steps involved in the translocation of lipids from the lumen of the small intestine through the enterocyte into the lymphatic system. Chapter 4 subsequently describes the effects of different lipid vehicles and formulations on the lymphatic transport of lipophilic drugs which gain access to the systemic circulation primarily via the intestinal lymphatics. Although the process of lipid transport is complex and not fully understood, an appreciation of the factors involved is fundamental to consideration of the absorption and transport of lipophilic molecules.

The American diet consists of approximately 100 to 200 g triacylglycerol (TG) per day,[1] although even greater amounts are consumed by certain population groups such as the Inuits (300 g/d). In all settings thus far studied, 95% of the ingested lipid is absorbed, including even those studies in which larger amounts of lipid were consumed during experimental investigations (500 g/d).[2] This efficient absorption and transport process is even more remarkable considering the fact that absorption normally occurs in the most proximal regions of the intestine.[3,4]

Because of the poor solubility of TG in water, the processes by which lipids are absorbed are complex. These complexities include the following aspects: (1) TG lipids do not mix with water and therefore must be rendered into a chemical form in which there is some ability of the product lipids to interact with the aqueous environment; otherwise, absorption would be greatly impaired. (2) The digested lipids must be resynthesized to TG in the enterocyte; otherwise, the absorbed fatty acids (FA) would traverse the intestinal absorptive cell and enter the portal vein for transport to the systemic circulation. The large amount of FA expected under these conditions would most likely damage the enterocytes, as well as possibly other tissues, due to their detergent properties. Furthermore, the FA would be taken up by the liver and either ß-oxidized or transported as very low density lipoproteins (VLDL). The end product of VLDL metabolism are low density lipoproteins (LDL) which are the major cholesterol transporter in the blood. (3) The resynthesized TG in the intestine must be transported to the general circulation which is accomplished by packaging the TG in a lipoprotein particle termed the chylomicron. This particle disperses the TG in a stabilized form by surrounding the TG with a monomolecular film of phospholipid, cholesterol, and specific apolipoproteins. Furthermore, the lipoprotein component enables the correct metabolism of the chylomicron in the periphery.

In addition to TG, the intestine also absorbs 2 to 2.5 gld/d phosphatidylcholine (PC) from dietary sources,[5] although a quantitatively more important source of PC is the bile. Approximately 11 g of biliary PC are presented to the intestine for absorption daily.[6] Additionally, 1 g of cholesterol is absorbed on a daily basis.

II. DIGESTION OF NEUTRAL LIPIDS

The digestion of neutral lipids is a very complex biochemical process occurring in the diverse mileu of the gastrointestinal tract. Factors that contribute to the complexity are the presence of (i) lipolytic enzymes (preduodenal, and pancreatic lipases) which act only at interfaces, (ii) a coenzyme (colipase) that facilitates the binding of pancreatic lipase to interfaces, and (iii) a variety of other necessary compounds and enzymes which include bile salts, phospholipids, pancreatic secretory trypsin inhibitor, cholecystokinin, and secretin.

A. PREDUODENAL LIPASE

All animal species thus far studied except for the chicken have preduodenal lipase active against TG,[7]although the site of synthesis is species dependent.[7] For example, rodent lipolytic activity is localized to the circumvallate papillae, whereas in polygastric species such as the calf and the sheep the preduodenal lipolytic activity is located in the glosso-epiglottic area. In other species which have been studied such as the dog, horse, pig, baboon[7] and man,[8] the preduodenal activity is located in the stomach where the lipase is produced in the chief cells.[9] As might be expected for these similar enzymes, considerable sequence homology exists amongst preduodenal lipases of various species, which in turn, differ greatly from that of the pancreatic enzyme.[9] The total activity of preduodenal lipase in various species is less than the total lipolytic activity derived from the pancreas and is estimated to be of the order 1:30 to 70, preduodenal lipase to pancreatic lipase activity.[7]

Since most of the activity of preduodenal lipase occurs in the stomach, it would be important for the pH-activity profile of the enzyme to be appropriate for an acidic environment. The pH optimum for preduodenal lipase in most species, including man, is between pH values of 4 and 6.[8,9] Preduodenal lipase has been shown to resist degradation due to either low pH (pH 1.5 to 2 for 2 h in monogastrics)[7] or exposure to pepsin (human and rabbit lipases).[9]

As a practical matter, the activity of preduodenal lipase is unimportant distal to the duodenum since pancreatic lipase is secreted with overwhelmingly more activity and the intestinal pH rises to neutrality where preduodenal lipase is inactive. In chronic pancreatitis or cystic fibrosis, however, the activity of preduodenal lipase may be prolonged because of the lack of alkalinization of the duodenum.[10] Under these conditions, the preduodenal lipase can be quite effective in hydrolyzing dietary lipid and can account for greater than 50% of lipid absorption.[11]

The hydrolytic site of the enzyme is predominantly at the sn-3 position of the TG molecule.[12] The resulting FA aids in the emulsification of the TG lipid and the increased surface area available for digestion increases the efficiency of digestion by pancreatic lipase within the small intestine.[13] Unlike pancreatic lipase, preduodenal lipase can attack TG even in the presence of an excess of

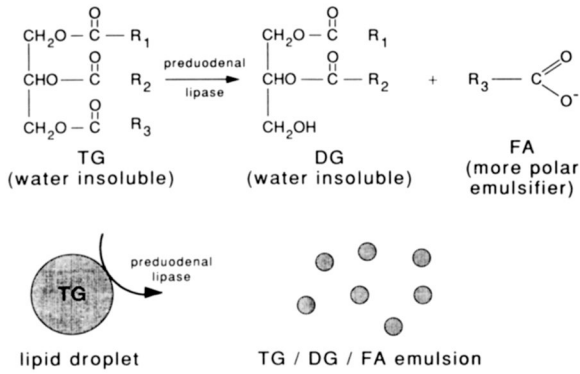

FIGURE 1. The action of preduodenal lipase is shown. In the superior part of the figure, the chemical reaction is schematically displayed. TG (triacylglycerol) which is water insoluble is hydrolyzed at the sn-3 position by preduodenal lipase producing diacylglycerol (DG) and fatty acid (FA). The fatty acid, which is more polar than either the DG or TG, acts as an emulsifier. This is schematically shown in the bottom of the figure where a large TG droplet is acted upon by preduodenal lipase. The FA is bound at the aqueous-lipid surface and, as an emulsifier, breaks up the large DG-TG droplets into smaller ones, thereby increasing the surface area of the lipid-water interface. A second fatty acid can be released by the preduodenal lipase, but this is an uncommon event under normal circumstances.

PC.[9] Figure 1 depicts the action of preduodenal lipase on ingested TG lipid.

B. PANCREATIC LIPASE

Lipases from the human, cow, sheep, and pig have been isolated[14–17] and have been shown to be 46 to 50 kD proteins. The primary structure of porcine lipase has been determined[18] and the human and dog lipases have been cloned.[19,20] The N-terminal 30 amino acids have been sequenced from human and porcine enzymes and they demonstrate considerable homology.[21]

Lipase activity is affected by bile acids. Bile acids can greatly inhibit lipolytic activity when present in concentrations either above[22] or below[23] their critical micellar concentration (CMC). The presence of bile acids also affects the pH optimum of pancreatic lipase. In the rat, the pH optimum is 8.0 in the absence of taurocholate and decreases to a value of 6.7 in the presence of 2 m*M* of the bile acid.[24]

Lipase is known as an "interfacial enzyme" as its activity is critically dependent on the presence of oil-water,[25] micellar[26] or air-water[27] interfaces. Lipase is inactive in the absence of an appropriate interface. The ability of lipases to penetrate an interface, as represented by a monomolecular lipid film, is dependent on the lateral surface pressure of the film and the species of origin of the lipase. Human lipase can penetrate and hydrolyze lipids at surface pressures of up to 30 dynes/cm,[21] whereas much lower pressures are required

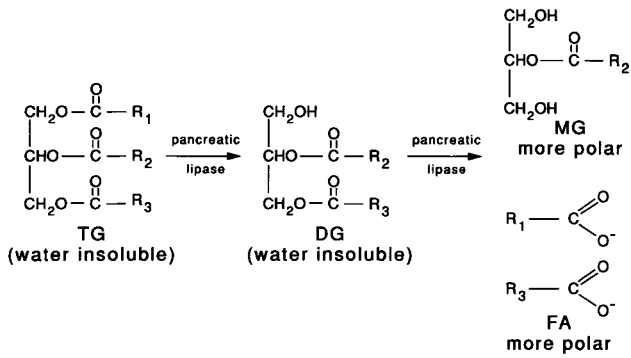

FIGURE 2. The hydrolysis of triacylglycerol (TG) by pancreatic lipase. TG is progressively hydrolyzed, first at the sn-1 and subsequently at the sn-3 position to initially produce DG (diacylglycerol) and a fatty acid (FA), and subsequently MG (monoacylglycerol) and another FA. Note that the MG and FA are both more polar than either TG or MG. The FA esterified at the sn-2 position is not hydrolyzed, and thus, MG and FA are the final products.

for porcine lipase to demonstrate activity.[21,27] Another facet of the interaction of lipases with interfaces is the ability of interfaces to inactivate the enzyme.[28]

Pancreatic lipase has a strict preference for the primary alcoholic groups of TG[29] and the reaction sequence is depicted in Figure 2. The resulting sn-2-monoacylglycerol (MG) may be absorbed as such, or it may slowly isomerize to the sn-1 isomer which then becomes susceptible to hydrolysis. However, absorption of MG appears to occur before significant amounts of isomerization. Lipase activity in the pancreas can be increased by a fatty diet[30] and results from augmented circulating levels of the gastrointestinal hormone, secretin.[31]

C. CO-LIPASE

Co-lipase is a 10 kD protein[32] of known sequence[33] which is found in the pancreas and is secreted in the pancreatic juice as a pro-enzyme.[34,35] The zymogen is cleaved by trypsin in the intestinal lumen to release the pentapeptide, Val-Pro-Asp-Pro-Arg.[34] The removal of the activation peptide enhances the ability of co-lipase to penetrate the phospholipid surface of emulsified TG.[35] A newly discovered property of the activation peptide is its ability to reduce the desire for food when injected intraperitoneally or fed to rats.[36]

In the absence of bile salts, co-lipase increases lipase activity several-fold and appears to penetrate the lipid film and help anchor the lipase to the interface,[27] which is reflected in a high binding constant for lipase (3.6×10^{-6} M).[37] In the presence of bile acids at a concentration above the CMC, co-lipase prevents the desorption of lipase from the interface that would otherwise occur thereby enabling hydrolysis to continue.[38] Without co-lipase, lipase is inactive in the presence of bile acids above their CMC.[22] Co-

lipase also improves the ability of lipase to penetrate lipid films at higher surface pressure.[27]

Lipase and co-lipase concentrations can be reduced in the intestine in chronic pancreatitis in which at least 90% of the gland must be destroyed prior to clinical symptoms becoming apparent.[39] Lipase activity may also be effectively reduced in the Zollinger Ellison Syndrome[40] in which a gastrin producing tumor results in gastric hyperacidity and consequently the acidic milieu created in the duodenum leads to inactivation of the lipase.

D. BILE ACIDS

Bile acids are normally secreted into the intestine as glycine or taurine conjugates of the two primary bile acids, cholate and chenodeoxycholate, and one secondary bile acid (deoxycholate) which is formed by bacterial 7α-dehydroxylation of cholate. Bile acid concentrations in the gall bladder are on the order of 120 m*M*.[41] When secreted into the intestine, dilution from the meal and gastrointestinal-pancreatic secretions result in a normal postprandial intraduodenal concentration of approximately 10 m*M*.[42]

Bile acids function as emulsifiers, emulsion stabilizers and solubilizers of lipids in the intestine. The solubilizing mechanism for lipids is the mixed micelle which is composed of multiple bile acid molecules whose hydrophobic sides face one another and whose hydrophilic sides face the aqueous environment.[43] Each bile acid forms micelles at a specific concentration, the CMC. The CMC depends on the hydrophobicity of the bile acid in question, the ionic strength of the medium, and the pH of the medium. When the various bile acids are mixed together, as they are in the intestine, their effective CMC is approximately 1.4 m*M*.[44] Bile acids will solubilize the lipid digestion products, but not the parent TG or diacylglycerol (DG) as these lipids only interact weakly with bile acid micelles. In the intestine, the solubilized lipid digestion products would be FA and MG resulting from the action of lipase on dietary TG. The stoichiometry of the association of the bile acids and FA is 1:1.4, respectively.[42]

The solubility of bile acids in the intestine depends on luminal pH and whether or not the bile acid is conjugated. For example, taurine conjugates have a very low pK_a and glycine conjugates have a pK_a in the range 4.3 to 5.0, whereas the constituent bile acids have pK_a values of 6.5 to 7.0.[43] At the pH normally present in the duodenum of 5.5 to 6.5,[42] unconjugated bile acids will be predominantly protonated, the glycine conjugates partially protonated, whereas the taurine conjuges will remain ionized. Protonated bile acids may be absorbed passively in the upper intestine, consequently reducing their concentration. The ionized bile acids are not absorbed proximally, but pass to the distal ileum where they are then absorbed by an active transport process.[45] In the ileum, the bile acids are absorbed into the ileal enterocytes by a high capacity, relatively low affinity transporter in the brush border membrane and exit by a high affinity transport system localized to the basolateral membrane.[46]

Bile salt concentrations can be reduced in the intestine by removing the site of active transport as in ileectomized individuals,[47] either intra- or extrahepatic obstruction preventing adequate bile flow to the intestine,[48] or diseases such as gluten-sensitive enteropathy in which the duodenal store of cholecystokinin is reduced and thus the gall bladder is not adequately contracted on the presentation of fat or protein to the intestine.[49] Finally, in the Zollinger Ellison Syndrome, the bile acids are protonated if the duodenal pH is low enough and then absorbed passively.

E. PANCREATIC SECRETORY TRYPSIN INHIBITOR

Pancreatic secretory trypsin inhibitor is a small 6 kD protein which is homologous with other Kazal type inhibitors and inhibits tryptic activity.[50] It is secreted in the pancreatic juice and has the property of promoting cholecystokinin release. Cholecystokinin causes contraction of the gall bladder and stimulation of secretion of digestive enzymes from the pancreas into the intestine. It is sensitive to tryptic activity and thus may serve as a "monitor peptide" in that the more protein in the intestinal lumen, the more peptide will be active since trypsin will be more likely to attack other proteins. Conversely, with little protein in the lumen, trypsin would rapidly attack and inactivate this peptide.

F. CHOLECYSTOKININ

As defined above, cholecystokinin (CCK) is a 33 amino acid peptide whose major action is to trigger the contraction of the gall bladder and stimulation of secretion of digestive enzymes from the pancreas into the intestine. It also causes the sphincter of Oddi to relax, enabling the bile and pancreatic juice to reach the duodenum. The minimal structure for complete expression of activity is the desulfated C-terminal heptapeptide.[51] The N-terminal 4 amino acids can, however, cause maximal stimulation of amylase release from pancreatic acini. Multiple forms of the hormone circulate.[52]

CCK is maximally localized to the duodenum where it is stored in endocrine-type cells. It is released by peptides, essential amino acids and hydrolyzed lipids.[52,53] Whole proteins and nonessential amino acids do not induce the release of CCK.[54] Fasting levels are on the order of 0.9 p*M*[55] and in response to a meal, levels of CCK increase to approximately 9 p *M*.[55] Bile acids that are released into the intestine in response to CCK participate in a feed back control loop in which CCK release is inhibited by their presence in the duodenum.[56]

G. SECRETIN

Human secretin is a 27 amino acid peptide[57] with a primary role in the potentiation of CCK activity[58] and causes the elaboration of bicarbonate rich fluid by the pancreas.[59] It also increases the flow of bile from the liver. Its effects on pancreatic enzyme output are controversial; it appears to stimulate

amylase release, but only at high concentrations.[60] It is found in maximal concentrations in the duodenum from which it is released at intraluminal pH values less than 4.5 in dogs,[61] and perhaps at lower pH values in humans.[62] Its action is mediated by the generation of cAMP.[63] Fasting concentrations are in the range of 2 to 4 p*M* and the plasma $t_{1/2}$ is approximately 3 min.[60]

H. LIPID AND BILE ACID INTERACTIONS IN THE INTESTINAL LUMEN

The major lipid form in which fats are ingested are as triacylglycerol. In this molecular state the lipid is essentially water insoluble and consequently is not absorbable in this form. This lack of absorption is demonstrated by the large amounts of lipid excreted in the stools in the absence of adequate lipolytic activity.[64] The lipolysis of TG results in the formation of more hydrophilic products, FA and MG (see Figures 1 and 2). The large lipid droplets that form in the stomach during the digestion of a meal or the intake of lipid formulations are partially broken into smaller droplets by the emulsification properties of the FA produced by preduodenal lipase. As the smaller droplets enter the duodenum, the luminal pH rises to 5.5 to 6.5, helping to ionize the FA, and bile acids are present in high, 10 m*M*, concentrations. These factors reduce the size of the lipid droplets further and thus greatly increase the surface area available for lipolytic attack.

Most of the information regarding the dynamic progress of the lipid phases formed during digestion come from observations made under the microscope using purified components. The first phase that forms in the presence of calcium ions and unsaturated bile acid micelles is a solid crystalline phase that occurs on the outside of the droplet.[65] This is composed of calcium soaps of the FA; no MG is present. No similar phase is created when rat lingual lipase is utilized in place of the pancreatic enzyme.[66]

As digestion proceeds, the surrounding calcium ion concentration becomes depleted, the pH of the medium drops, and the local MG concentrations rise. All these events reduce the formation of the solid crystalline phase and result in the production of a viscous isotropic phase or liquid crystalline phase.[67] In addition to these product phases, vesicles and lipid-rich mixed micelles are also formed. All of these products are the result of the progressively greater interaction of the produced FA and MG with water. It should also be clear that as the micelle becomes depleted of lipid due to absorption, micellar lipid is replaced by lipid from vesicles and in turn vesicular phase lipid is repleted from the liquid crystalline phase. These events are schematized in Figure 3.

III. ABSORPTION OF NEUTRAL LIPIDS

Following lipolysis, the vesicles and mixed micelles containing the end product phases of lipid digestion must then traverse an unstirred water layer to

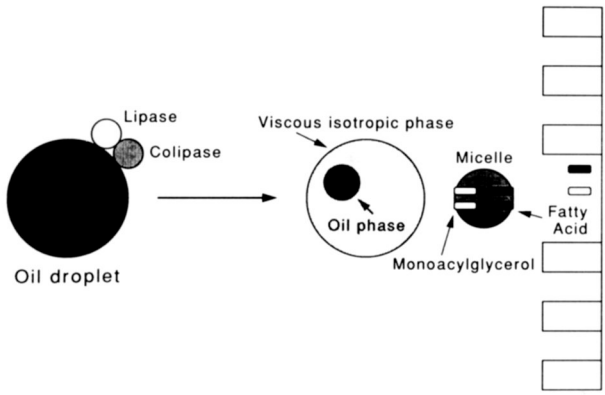

FIGURE 3. An oil droplet is schematically shown to be undergoing hydrolysis. Lipase and colipase are bound to the surface at the oil-water interface. Hydrolysis first produces a solid crystalline phase which is not shown. Further hydrolysis produces a viscous isotropic phase and residual oil phase as indicated. Completion of hydrolysis produces the bile acid mixed micelle containing fatty acid and sn-2-monoacylglycerol. Both the monoacylglycerol and fatty acid are shown approaching the brush border of the enterocyte.

reach the surface of the intestinal absorptive cells.[68] This layer is present at the surface of membranes and presents a diffusion barrier to the absorption of long chain FA and other lipid digestion products. While it is comparatively easy to control the thickness of the unstirred water layer during *in vitro* experiments by varying the rate of stirring or fluid flow through the intestinal tract, it is more difficult to assess its effective thickness *in vivo*. Since the rate of diffusion is an inverse function of the radius of the diffusing specie, squared, the larger mixed micelles will diffuse more slowly than monomeric lipids. Nevertheless, the greater concentration of lipids in the mixed micelles as compared to their monomeric concentration in the aqueous phase (see Table 1) makes the micelles an important component of the absorptive process.

Figure 4 depicts the approach of FA to the microvillus membrane and its proposed entry into the enterocyte. The pH of the lumen in the proximal jejunum is around neutrality, resulting in ionization of the FA. Next to the brush border membrane, however, the pH falls to appoximately 6.0.[69] This results in a greater fraction of the FA being protonated, thereby helping its passive entry through the microvillus membrane. In addition to the passive movement of FA into the cell, a new transporter has been proposed which is termed the FA transport protein or FATP. This protein, currently called the membrane fatty acid binding protein, is a 40 kD protein associated with brush border membranes.[70] The FATP demonstrates saturation kinetics with a K_m of 93 nM and a V_{max} of 2.1 nmol/min/10^6 cells.[71] The physiological importance of the protein is suggested by the inhibition of FA uptake by antibodies specifically directed against it.

TABLE 1
Concentrations of Various Lipid Digestion Products in Intestinal Fluids Present in either Monomeric or Micellar Phases after a Meal

Lipid fraction	Monomer (m*M*)	Micelle (m*M*)
Monoacylglycerol	0.12	4.6
Fatty acid	2.3	54.8
Lysophosphatidylcholine	0.18	6.4
Cholesterol	0.01	2.4

Note: The study was conducted in normal human subjects who were intubated to the ligament of Trietz after an overnight fast. The ingested meal consisted of two fried eggs, bacon, toast with butter, and milk.

From Mansbach, C. M., II, Cohen, R. S., and Leff, P. B., *J. Clin. Invest.*, 56, 781, 1975. With permission.

In sum, the appropriate release of the digestive hormones in response to a meal, the rapidity with which pancreatic lipase operates, and the ability of the bile acid micelles to "solubilize" hydrolytic products result in an efficient absorptive system.[4] The efficiency of the absorptive process is depicted in Figure 5 where up to 80% of the radiolabeled triolein contained in a 20 g corn oil liquid meal was absorbed within 60 cm distal to the pylorus.

IV. INTRACELLULAR PROCESSING OF LIPID DIGESTION PRODUCTS

Once inside the enterocyte, the FA and MG must be desorbed from the microvillus membrane and transported to the endoplasmic reticulum where resynthesis to TG occurs. It is unclear whether this process is aided by the intracellular FA binding protein (FABP), although the FABP can deliver FA to model membranes[72] and from liposomes to microsomes *in vitro*.[73] This 12 kD protein, including the discovery that there are both hepatic and intestinal forms with differing binding profiles for FA,[74] has been cloned and expressed.[75] However, no role has been clearly defined for FABP with respect to its original putative function. The other potential mechanism for the desorption of the FA and MG is diffusion down concentration gradients as the cytosol becomes depleted of both lipids due to their rapid metabolism by enzymes present in the endoplasmic reticulum.

Studies using microsomes have shown that FA and MG are quickly taken up and converted to TG by either of two synthetic pathways. These are depicted in Figures 6 and 7 and are commonly referred to as the monoacylglycerol and α-glycerophosphate pathways, respectively. The monoacylglycerol pathway schematized in Figure 6 is the simplest and is preferred if there is MG present.

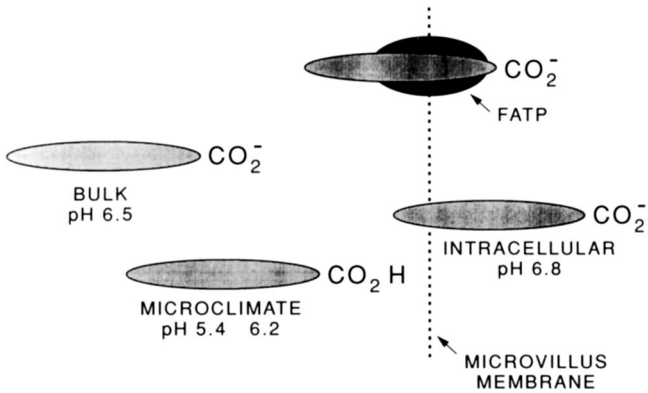

FIGURE 4. The passage of a fatty acid (FA) through the microvillus membrane. An ionized FA is schematized in the intestinal lumen where the pH is approximately 6.5. The FA becomes protonated in the acidic microclimate adjacent to the microvillus membrane. The FA can pass passively into the cell or it may be actively taken up by the fatty acid transport protein (FATP). Once inside the cell, the higher pH causes the FA to become ionized, thereby trapping it intracellularly.

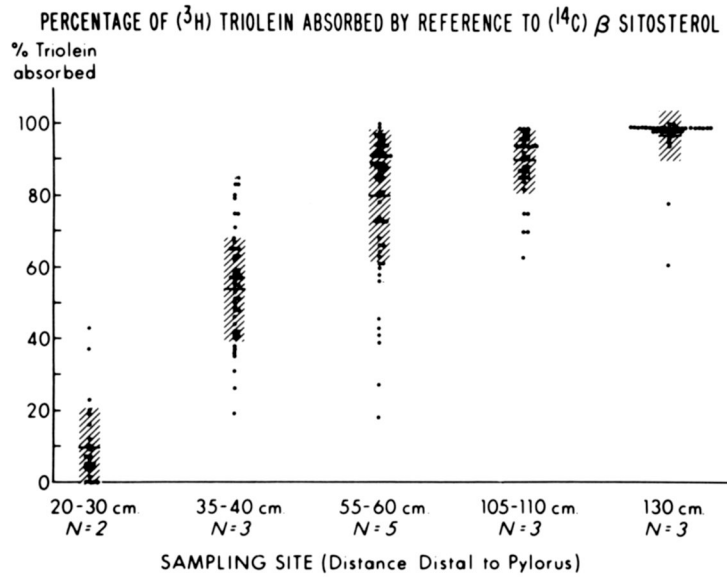

FIGURE 5. The percentage absorption of [^{3}H]-glyceryl trioleate using [^{14}C]-ß-sitosterol as a nonabsorbable marker. A blenderized liquid meal consisting of 20 g corn oil, 2 raw eggs, 63 g skim milk powder, and 69 g dextrose in 500 ml water labeled with the radiolipids was fed to six healthy males after intubation with a double lumen tube. Samples were obtained every 15 min for a 4 h-period. In three subjects, samples were obtained 35 to 40 cm and 105 to 110 cm distal to the pylorus, and in the other three subjects samples were obtained at 60 and 130 cm distal to the pylorus. The percentage absorption was calculated as $[1-(^{14}C/^3H \text{ meal})/(^{14}C/^3H \text{ sample})]\times 100$. (From Newton, D. F. and Mansbach, C. M., II. *Clin. Chim. Acta*, 89, 331, 1978. With permission.)

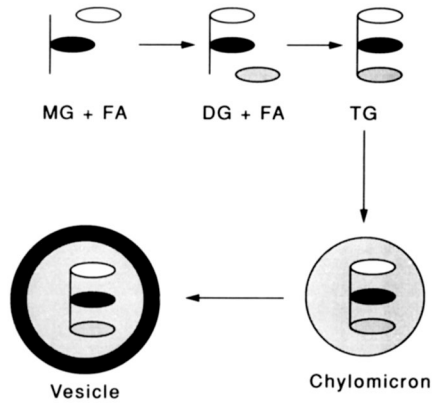

FIGURE 6. A schematized presentation of the MG (monoacylgylcerol) pathway of TG synthesis. In the upper left of the figure, sn-2 MG is acylated by FA-CoA at the sn-1 position. This produces DG (diacylglycerol) which, in turn, is acylated by a second FA-CoA at the sn-3 position producing TG. The TG is shown in the interior oily portion of the chylomicron. The chylomicron combines with other vesicles which ultimately fuse with the basolateral membrane of the intestinal cell enabling the chylomicrons to enter the lamina propria and ultimately the lymphatics.

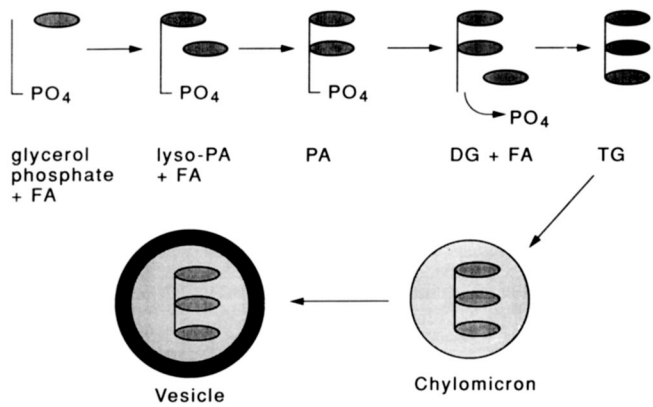

FIGURE 7. This is a schematic of the *de novo* pathway of triacylglycerol (TG) synthesis. In the upper left of the figure, sn-3 glycerolphosphate is esterified with fatty acid (FA) at the level of its Co-A derivative. This produces sn-1 lysophosphatidic acid (lyso-PA). The lyso-PA is acylated with FA-CoA to produce phosphatidic acid (PA). Phosphatidic acid phosphatase dephosphorylates the PA to produce diacylglycerol (DG) which is acylated with FA-CoA to produce TG. This TG forms the lipid center of chylomicrons which combine in vesicles and eventually fuse with the basolateral membrane as in Figure 6.

In the intact intestinal cell, this pathway appears to signal that the TG formed will exit the cell as a component of chylomicrons.[76] The monoacylglycerol pathway has a fast turnover[76] and is the most conserving of ATP units.

The α-glycerophosphate pathway illustrated in Figure 7 utilizes *de novo* synthesized glyceride-glycerol as a precursor. This pathway has a slower turnover rate than the monoacylglycerol pathway and there are indications that TG produced from this pathway may not exit the intestine solely in lymph, as levels have been observed in the portal vein and there may also be some efflux back into the intestinal lumen.[76–78] The enzymes responsible for both pathways have been shown to also be present on the cytosolic surface of liver microsomes[79,80] and presumably this is also the case with the enterocytes. The activity of at least one of these enzymes is greatest in the villus tips of the proximal intestine.[81]

In the monoacylglycerol pathway, fatty acid CoA synthetase utilizes FA, ATP, and CoA to subsequently produce FA-CoA, AMP, and pyrophosphate.[82] Monoacylglyceride acyltransferase then utilizes sn-2-MG and FA-CoA to produce the corresponding sn-1,2-diacylglycerol.[83] The intestinal monoacylglyceride acyltransferase acylates MG esterified at the sn-1 position[83] and appears to be different from an enzyme of similar function present in the neonatal liver of rats.[84] The liver enzyme will not acylate sn-1-MG.[83] Furthermore, the intestinal enzyme is more thermolabile, more resistent to diethylpyrocarbonate and trinitrobenzene sulfonic acid than is the hepatic enzyme.[85] The final enzymatic step in the intestinal synthesis of TG involves diacylglycerol acyltransferase which utilizes sn-1,2 DG and FA-CoA.[81] As noted previously, DG acyltransferase is most likely located on the cytosolic surface of the endoplasmic reticulum and thus its product is similarly situated. The TG must then flip to the luminal side where it remains within the interior of the ER-Golgi membranes until it enters a secretory vesicle preparatory to its exit by reverse pinocytosis at the basolateral portion of the enterocyte.[86]

The α-glycerophosphate or *de novo* pathway of TG resynthesis utilizes sn-3-glycerol phosphate (G3P) formed from either glucose metabolism or the transamination of amino acids. The intestine, however, phosphorylates glycerol poorly.[87] Entry into this pathway is also from FA-CoA formed from FA-CoA ligase. Glycerol-phosphate acyl transferase acylates G3P resulting in the formation of lyso-phosphatic acid (lyso-PA) which then undergoes acylation via lysophosphatidic acid acyl transferase to form phosphatidic acid (PA). The phosphate group is then removed by phosphatidic acid phosphatase to produce the corresponding DG. This enzyme is thought to be rate limiting in the *de novo* TG synthetic pathway.[88] Although not well studied in the intestine, in the liver the phosphatase is known to have both a cytosolic and a bound state.[89] The bound form of the enzyme is active and its translocation from cytosol to bound state is stimulated by the presence of FA. The DG formed by the dephosphorylation reaction is neither chemically or metabolically equivalent with the DG

formed from the acylation of MG. The DG synthesized from MG is only able to be acylated to TG, whereas the DG synthesized from PA may either form TG or phosphatidylcholine (PC).[81,90] The DG is acylated to TG by diacylglycerol acyl transferase.[81] The TG so formed is translocated to the luminal side and thus becomes part of the chylomicron precursor TG. The enterocyte is not like the hepatocyte with regard to the potential for β-oxidation of FA since it performs this function poorly.[91] Thus, most of the FA presented to the intestines are esterified to TG.

V. DIGESTION OF PHOSPHOLIPIDS

Phosphatidylcholine (PC), often referred to as lecithin, is the principle phospholipid ingested and found in the body and its absorption and synthesis is discussed here as an example of how phospholipids are handled by the intestine. In terms of lipid transport, phospholipids play a key role in stabilizing the surface properties of lipoproteins (primarily chylomicrons) produced by the enterocyte. Prior to absorption, PC requires the removal of an acyl group to form lyso-PC or lyso-lecithin.[92] Because of the stereospecificity of the pancreatic phospholipase which performs this function (phospholipase A_2),[93] phospholipids are absorbed in the sn-1 lyso form. Both the FA and lyso-PC products have greater water solubility than the original PC and appear to be readily absorbed. Figure 8 depicts the metabolism of PC in both the lumen and the enterocyte. The majority of the PC absorbed each day in humans comes from the bile, which contributes about 10 g,[6] whereas the diet contributes only about 2 g daily.[5] The rate of PC secretion in the rat is approximately 12 to 14 μmol/kg/hr.[94]

Phospholipase A_2 is synthesized in the pancreas as a zymogen[95] with activity against monomeric PC. However, the activity does not increase in the presence of lipid-water interfaces which would normally be present in the postprandial intestine.[96] Trypsin hydrolysis removes the first seven amino acids, resulting in an enzyme with markedly increased activity at oil-water interfaces.[96] Phospholipases, like lipases, are operative only at certain surface pressures. At surface pressures that are either too high or too low, the enzyme has no activity. This can be shown using the monolayer technique in which the surface pressure can be carefully controlled. An example is shown in Figure 9.[97] The activity is lost due to the inability of the enzyme to penetrate the interface and gain access to its substrate. At high surface pressures, a lag phase exists[98] in which no activity occurs for a finite period of time followed by progressively rapid hydrolysis up to its maximum rate for the particular surface pressure applied. The lag phase can be eliminated by acylation of Lys-56 which may come from the substrate.[99]

In the intestinal lumen, conditions are such that phospholipolysis occurs. A high bile acid to phospholipid ratio promotes lipolysis.[42,100] Calcium ions are also present and are required for phospholipolysis to occur.[42] The pancreas also

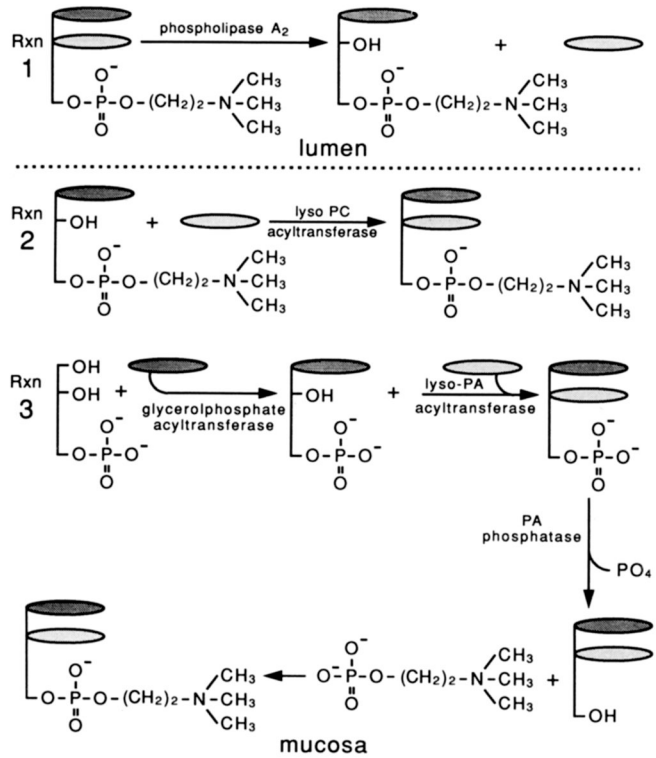

FIGURE 8. The metabolism of phosphatidylcholine (PC) in both the lumen and mucosa is shown. Reaction 1 (Rxn 1) takes place in the lumen through the mediation of phospholipase A_2 in which the sn-2 acyl group is stereospecifically removed from the PC. Reactions 2 and 3 take place in the mucosa. In reaction 2 (Rxn 2), lyso-PC is acylated at the sn-2 position by lyso-PC acyltransferase utilizing fatty acid acyl-CoA. Reaction 3 (Rxn 3) is the *de novo* pathway of PC synthesis. It begins with glycerophosphate which is acylated utilizing fatty acid-CoA to form lyso-PA. A second acyl group is added at the level of its CoA derivative to lyso-CoA to thereby form PA. PA is dephosphorylated and the reaction product, diacylglycerol (DG), has a phosphocholine group added at the level of CDP-choline. This addition is mediated by DG acyltransferase which produces PC.

synthesizes a phospholipase A_1[101] which hydrolyzes the acyl group at the sn-1 position of phospholipids. While this may also be secreted into the intestine during stimulation of pancreatic secretions, it appears not to have a physiological role during the digestion of phospholipids.

VI. INTRACELLULAR METABOLISM OF PHOSPHOLIPIDS

The sn-1 lyso-PC may be acylated by lyso-PC acyltransferase (Figure 8)[81] which utilizes FA-CoA as substrate to produce PC. The enzyme has its greatest specific activity in the villus tips of the proximal intestine.[81] It is an endoplas-

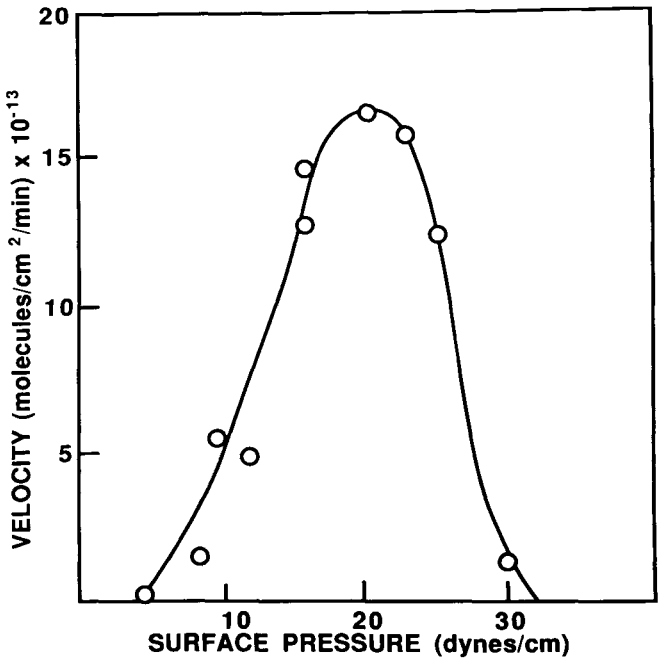

FIGURE 9. The activity of bee venom phospholipase A_2 as a function of surface pressure. The hydrolytic rate (in arbitrary units) is shown on the ordinate and the surface pressure at which the assays were performed on the abscissa. Bee venom phospholipase A_2 (0.91 μg) was injected below a monolayer composed of dinonanoyl PC. (From Mansbach, C. M. II., *Gastroenterology*, 98, 1369, 1990. With permission.)

mic reticulum enzyme located on the cytosolic leaflet[79] and its activity in hamsters may be induced by fatty diets.[102] PC synthesized via this pathway is preferred as a precursor for chylomicron PC, but is limited by substrate availability.[103]

PC can also be synthesized *de novo* (Figure 8). Synthesis starts with glycerol-3-phosphate, G3P, which is acylated by glycerol phosphate acyl transferase. It uses FA-CoA to produce lyso-phosphatidic acid (PA). Lyso-PA is acylated to PA again by an acyl transferase (lyso-PA acyl transferase) which utilizes FA-CoA. The PA must then be dephosphorylated by PA phosphatase as described previously. The resultant DG is then able to act as an acceptor for phosphocholine which is mediated by CDP cholinephosphotransferase utilizing CDP-choline as the substrate. This enzyme is responsive to fat diets,[102] is equally distributed between villus tips and crypts and is evenly distributed throughout the intestine.[81]

In this PC synthetic pathway, the synthesis of the substrate CDP-choline appears to be the rate limiting step.[104] CDP-choline is formed via a two step reaction beginning with choline phosphate, synthesized by the cytosolic en-

zyme, choline kinase and CTP. Choline phosphate and CTP are utilized as substrates for CDP-choline cytidylyltransferase which catalyzes the synthesis of CDP-choline. This enzyme is present in both the cytosol and at the endoplasmic reticulum,[105] although only the ER form is active. The enzyme appears to translocate between cytosol and its bound form depending on PC synthetic requirements and its activity can be controlled by phosphorylation.[106] In isolated hepatocytes, TG transport is limited by the activity of the cytidylyltransferase.[107]

Adequate availability of PC for chylomicron formation is an important factor in lymph TG transport. For example, the inclusion of PC in TG infusions can increase the lymph TG output by a twofold factor in intact rats,[76] and there are indications that the *de novo* pathway of PC synthesis may not be adequate for maximal TG transport.[108]

VII. CHOLESTEROL AND CHOLESTEROL ESTER ABSORPTION AND INTRACELLULAR METABOLISM

Cholesterol is ingested both as the free and esterified form in amounts that vary with the diet. The esterified sterol requires hydrolysis (cholesterol esterase) prior to absorption. Unlike neutral and phospholipid absorption, cholesterol absorption is only about 50% of intake.[109] Cholesterol esterase is synthesized in the pancreas as a 82 kD protein[110] and transported to the duodenum with other pancreatic enzymes. Of the different bile salts, only taurocholate promotes the activity of the enzyme.[111] Bile acids are required for cholesterol absorption and although cholesterol is poorly "solubilized" in pure bile acid micelles, its solubility is considerably enhanced in the presence of MG and FA.[112] Its rate of absorption appears to be less than MG and FA so that as the mixed micelle becomes depleted of these neutral lipids, the rate of sterol absorption slows appreciably.[112]

The intracellular metabolism of cholesterol is complex. The enterocyte is the only cell that receives sterol from three different but interrelated sources, i.e., the lumen, intracellular synthesis, and from LDL via specific receptors on the basolateral membrane.[113] Interrelationships exist between these three sources because an increase in cholesterol absorption will result in a reduced rate of cholesterol synthesis.[114,115] By contrast, when cholesterol absorption is inhibited, cholesterol synthesis is increased.[116] Similarly, increased cholesterol taken up by the enterocyte from LDL reduces cholesterol synthesis.[115] As in other cells, the regulatory enzyme of cholesterol synthesis is 3-hydroxy-3-methylglutaryl coenzyme A reductase.[115]

VIII. LIPOPROTEIN METABOLISM

Apolipoproteins play an important role in terms of chylomicron transport. However, the intestine synthesizes only some of the apolipoproteins present in

chylomicrons. Synthesis of apolipoprotein B (apo B) by the intestine is clearly of major importance in terms of chylomicron transport since the congenital absence of its synthesis results in no chylomicron formation.[117] Even the intravenous replacement of apo B does not restore TG transport to normal[118] although hepatic, and not intestinal apo B, was utilized in the study. Intestinal apo B (apo B-48) is 48% of the molecular weight of the apo B synthesized in the liver (apo B-100) as a 520 kD protein. The so-called apo B-48 is genetically equivalent to apo B-100 except that it contains a unique stop condon truncating the mRNA transcribed.[119]

Surprisingly, regulation of apo B-48 synthesis does not appear to be dependent on diet,[120] and in fact, bile diversion in the fasting rat model is the only probe that has been shown to alter its synthesis.[120] In this model, if bile is repleted, apo B-48 synthesis is restored to normal. Both the PC and bile acid components of bile appear to have major effects in restoring synthesis to normal rates.[121]

In the rat, the intestine synthesizes approximately 16% of the total body synthesis of apo B, 56% of apo A-I, and 59% of apo A-IV but little of the other apolipoproteins.[122] As such, it is clearly an important contributor to the pool of circulating lipoproteins. In the absence of protein synthesis, for example in the acetoxycycloheximide treated rat, lipid and chylomicron transport is significantly inhibited.[123] The chylomicrons that are formed are larger than normal suggesting the importance of the apolipoproteins to chylomicron surface stability and, perhaps, transport.

IX. FLOW OF NEUTRAL LIPIDS THROUGH THE ENTEROCYTE

There appear to be at least two pools of TG in the enterocyte as judged from data in the steady state, intraduodenally infused TG, lymph fistula rat model.[78] In this system, the specific activity of the TG in the lymph approaches that of the TG infused, while at the same time the TG in the mucosa is only half that of the infusate TG.[78] These two pools have been called the "fast" and "slow" TG pools according to their rate of turnover. The "fast" pool is composed primarily of TG synthesized from luminal MG. It has a fractional turnover rate of 0.6 per hour.[76] Newly synthesized TG rapidly enters the pool[124] and the TG from this pool exits the enterocyte to be transported in the lymph. The pool is expanded by the co-infusion of PC with TG.[76] The "slowly" turning over pool is composed primarily of *de novo* synthesized glyceride-glycerol, has a specific activity that is less than the infused TG,[76] is not transported into the lymph,[125] and may exit the cell either by refluxing into the lumen[78] or into the interstitium where it is eventually transported via the portal vein.[77] TG enters this pool slowly.[124]

Inside the enterocyte there are both acidic and alkaline lipases present. The acidic lipase has been purified as a 51 kD protein with a pH optimum

of 5.6.[126] The enzyme has its greatest activity in the villus tips of the proximal intestine suggesting a role in the metabolism of dietary lipids.[127] This is further suggested by the large lipid droplets seen in enterocytes in neonates who congenitally lack the enzyme (Wolman's disease).[128] However, its physiological importance is questionable since relatively small amounts of lipid accumulate in the enterocyte when the enzyme is inhibited during lipid absorption[129] and little hydrolysis is observed at acidic pH on incubation of the mucosa at various pH values.[124] The alkaline lipase may have physiologic importance since at alkaline pH values, TG in mucosal homogenates undergoes rapid hydrolysis even when the pancreatic duct has been ligated for 48 hr.[124] These lipases may hydrolyze TG which is not transported in lymph and the FA product may then be transported from the enterocyte in the portal vein.[77]

X. TRANSPORT OF TRIACYLGLYCEROLS INTO THE LYMPH

TG moves rapidly from lumen into lymph, a process which can occur within 14 min.[130] Therefore, it is readily apparent that the processes of luminal hydrolysis, mucosal reesterification and transport into the lymph as components of chylomicrons is fast despite the many steps involved. The lymph transport of TG can be increased by the co-infusion of PC with TG.[76,125] In the rat bile fistula model, TG transport is reduced compared to control, although the TG transport can be restored by the co-infusion of PC.[108] Supplementation of the infusate with choline is not sufficient to restore TG transport. These data suggest that the intestine may not be able to generate enough *de novo* synthesized PC to adequately facilitate the transport of available TG. Transport of TG is also reduced when the ground substance of the interstitium is dehydrated.[130] The triglyceride FA composition of lymph lipid closely reflects the acyl group composition of the luminal TG.[78,131,132]

XI. PORTAL VEIN TRANSPORT OF LIPIDS

The portal vein has been shown to transport approximately 15% of absorbed FA,[133] (especially unsaturated FA[134]) and recently a greater role for the portal vein transport of FA has been claimed.[77] In these particular studies, large amounts of TG were intraduodenally infused and 39% of the infused lipids were found to be transported in the portal vein.[77] Most of the transported lipid was in the form of TG although some FA was also present. Furthermore, it was found that large amounts of endogenously derived FA were also present in the portal vein indicating that the lipid absorptive process, in an undefined way, caused the elaboration of endogenous FA. It is most likely the FA were hydrolytic products of TG either from liver or fat deposits and were transported to the intestine.

XII. SUMMARY

This review has covered many, but by no means all, of the aspects of intestinal lipid absorption, metabolism, and transport. This complex field requires considerably more work before a clearer understanding emerges. Several points can be made. Dietary lipids, being insoluble in water, require hydrolysis prior to absorption. Bile acid mixed micelles and vesicles enable the solubilized products to rapidly penetrate the intestinal mucosa. These products must be resynthesized to TG prior to their transport for two major reasons. Firstly, the large amounts of FA presented for absorption, if not synthesized into TG, could potentially solubilize or otherwise disrupt cellular or intracellular membranes. Secondly, although outside the scope of this review, the lipoproteins on the surface of the chylomicrons provide information as to their disposal. Although the entire process takes many steps, these are, some would say, all too efficiently performed.

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Chapter 3

METHODOLOGIES FOR ASSESSING INTESTINAL LYMPHATIC TRANSPORT

Thomas J. Raub, Scott L. Douglas, George W. Melchior, William N. Charman, and Walter Morozowich

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I. ANIMAL MODELS FOR LYMPHATIC AND VENOUS DELIVERY STUDIES

A. INTRODUCTION

The potential advantages of transporting drugs through the intestinal lymphatic pathway include avoidance of hepatic first pass metabolism, the selective treatment of diseases of the mesenteric lymphatics, inhibition of cancer cell metastasis and a potential enhancement of the absorption of large molecules such as peptides and particulates.[1–6] Furthermore, lymphatic drug delivery offers the potential for lymphocyte targeting as well as receptor-mediated targeting via the low density lipoprotein receptor.[7–8]

The intestinal absorption of highly lipophilic compounds frequently leads to significant lymphatic delivery due to association of the compounds with the lipoprotein particles synthesized within the enterocyte. These lipoprotein particles carrying the associated drug are exocytosed from the enterocyte and travel through the mesenteric lymphatic system, eventually ending up in the systemic venous circulation. Poorly permeable compounds, such as highly polar drugs, macromolecules, and peptides can be forced into the lymphatic system by another approach initiated with opening of the tight junctions between the intestinal enterocytes. Once in the lamina propria, these poorly membrane permeable substances do not readily diffuse across the venous capillary and, as a result, they pass through the relatively large terminal openings of the lymphatic vessels leading to lymphatic delivery.[9]

The development of drug delivery systems based on the lipid uptake and lipoprotein pathways requires specialized methodologies from the pharmaceutical, biological, and synthetic chemistry disciplines. The objective of this chapter is to describe some of the methodologies which the authors have found useful in lipid/lipoprotein transport studies and in the development of lymphatic- or lipoprotein-based drug delivery systems. These methodologies include techniques for conducting lymphatic delivery studies in the conscious rat

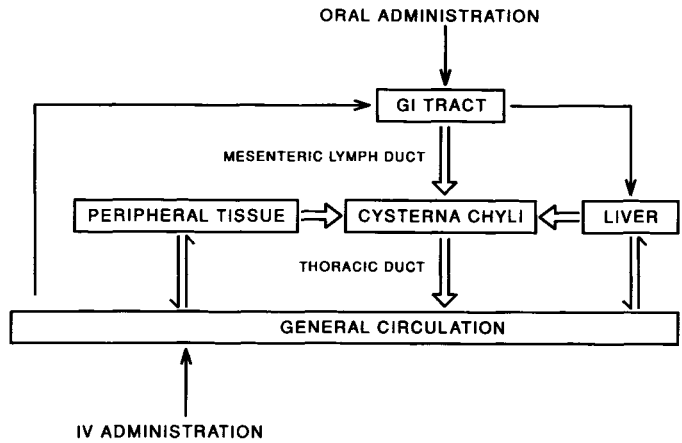


FIGURE 1. Schematic representation describing the different lymphatic duct cannulation sites and the potential for equilibration of drugs between the general circulation and the peripheral lymph.

and in the dog. In addition, methodologies are presented for separating lipoproteins and for mapping the cellular transport of drugs via the lipid and lipoprotein pathways using light and electron microscopy. Since the rat is widely used in lymphatic delivery studies,[10] a detailed procedure is presented for preparation of a conscious rat lymphatic delivery model based on cannulation procedures reported in the literature.[11–14]

B. METHODOLOGICAL DIFFERENCES ASSOCIATED WITH TRANSPORT STUDIES

There are numerous experimental methodologies and protocols which have been reported for the assessment of lymphatic transport in the rat. The most common differences between them include (i) the site of cannulation and lymph fistulation, (ii) the prepartion of the animal for lymph duct cannulation, (iii) the extent of hydration and the fasting/fed state of the animal after lymph duct cannulation, (iv) whether the experiment is performed in a conscious or anesthetized animal, and (v) the site of drug/lipid administration.

Intestinal lymph can be collected by fistulation of either the mesenteric lymph duct or the thoracic duct. The mesenteric lymph duct drains lymph originating from the small intestine, whereas the thoracic lymph duct receives lymph (via the cisterna chyli at the distal end of the thoracic duct) from the small intestine, periphery, and the liver. Figure 1 represents the functional relationship between intestinal and thoracic lymph, and depicts the equilibration which can occur between the general blood circulation and the lymphatic system. The transfer of drugs between blood and peripheral lymph has been observed with a range of compounds including *p*-aminosalicylic acid,[15] isoniazid,[16] cylcosporin,[17] DDT,[18] and some lipophilic vitamins.[19]

The collection of thoracic lymph may lead to an over-estimation of the extent of intestinal lymphatic drug transport. This is because an orally administered drug which is absorbed via the portal blood and reaches the general circulation intact, may equilibrate with the peripheral lymph which then drains into the thoracic lymph.

Noguchi and co-workers[18] measured the levels of DDT appearing in either the mesenteric or thoracic lymph of appropriately cannulated conscious rats after oral administration of a 2 mg dose dissolved in oleic acid. The DDT recovery (% dose, Mean ± SE) was 32.3 ± 2.6% and 21.9 ± 2.3% in the thoracic and mesenteric lymph, respectively. The increased recovery of DDT in the thoracic lymph was due to equilibration of DDT between the general circulation and the peripheral lymphatics which subsequently drain into the thoracic lymph. These data indicate that mesenteric lymph duct cannulations are the preferred means of lymph fistulation for assessing intestinal lymphatic drug transport. However, for studies where equilibration of the administered compound between the general circulation and lymph is not likely (e.g., in lipid transport studies where lipid absorption via the portal blood is not likely to be significant), then either point of cannulation would be acceptable. A further complication which may be associated with interpretation of intestinal lymphatic transport data based on thoracic lymph levels is that the presence of lipoproteins within the lymph system may facilitate the exchange of drugs between the general circulation and the lymphatics.

The timing of lymph duct cannulation and the preparation of the animal for subsequent drug/lipid administration may affect the rate and magnitude of the observed lymphatic transport. For example, the period of fasting after surgery, the extent of hydration, and whether or not the studies are performed in anesthetized or conscious animals are potential variables. Although these factors are generally controlled for within a series of experiments in a particular laboratory, they are likely to be different between study centers and hence the comparison and extrapolation of data is often a difficult task.

In an anesthetized mesenteric lymph duct cannulated rat model, the intestinal lymphatic transport of DDT was increased by a factor of approximately twofold when the period of fasting after cannulation was varied between 2 and 48 hr.[20] The increased transport was possibly due to mobilization of endogenous lipids and increased lipoprotein synthesis.[21] Mansbach and co-workers have demonstrated that the pre-feeding of rats with triglyceride or phospholipid prior to studying lymphatic transport can increase the extent of lipid transport.[22,23] These observations should be considered when lipid is administered to aid visualization of the mesenteric lymphatics during surgery, and when the recovery period after surgery and prior to drug administration is limited.

The extent of prior hydration in the rat has been shown to influence the rate of appearance of lipoproteins in mesenteric lymph after administration of lipid.[24] As the lymphatic transport of lipophilic drugs is associated with the formation and transport of lipoproteins, these hydration-related effects would be expected to translate to effects on the rate of drug appearance.

In an anesthetized animal model the administration of a drug/lipid formulation is most often via a cannula implanted within the duodenum, whereas in a conscious animal model the drug/lipid formulation may be administered either orally or via an intestinal cannula. If administered orally, the formulation components may affect the rate of gastric emptying and hence the rate of presentation of the drug and lipid to the small intestine. Therefore, if it is necessary to estimate the intrinsic ability of the small intestine to lymphatically transport a drug or lipid, then administration of the formulation components is ideally performed via a duodenal cannula.

C. CANNULATION TECHNIQUES FOR THE MESENTERIC LYMPHATIC DUCT, DUODENUM, AND JUGULAR VEIN IN THE RAT

Since the rat is widely used in lymphatic delivery studies,[10] a detailed procedure is presented for the preparation of a conscious rat lymphatic delivery model based on different cannulation procedures reported in the literature.[11–14] A number of related conscious rat models have been reported and the original literature should be consulted for greater detail.[25–33] Intestinal perfusion models have also been reported[34] and these have utility in determining the extent of venous delivery by cannulating the mesenteric blood supply.[10,35] Alternatively, venous delivery can be determined by appropriate sampling of blood from the portal vein.[36–38]

The following section describes the development of a conscious triple-cannulated lymphatic rat delivery model employed in the authors' laboratories. The description is intentionally detailed to enable the experimental system, if required, to be replicated and established in laboratories which are not currently assessing intestinal lymphatic transport of lipids and drugs. This particular experimental system is one of many different potential approaches which may be employed and is representive of the approaches which may be taken. The model involves surgically implanting separate cannulas in the jugular vein, the duodenum (just below the pyloric valve), and the mesenteric lymphatic duct in the rat. Surgery is performed at two separate times with jugular surgery being done first and, following a recovery period of 3 to 4 days, the abdominal surgery is then performed. Normal saline is infused into the intestine immediately after abdominal surgery and continuing through the recovery and study periods in order to maintain hydration and provide for constant lymph flow. After allowing 48 hr for recovery, the animals are placed in restraining cages, at which time dosing and blood/lymph collection occurs.

1. Materials and Methods

A typical set of surgical equipment required for performing the described surgery would consist of the following: 1.5 inch straight scissors, straight Kelly forceps (size 8), Backhaus tared forceps, Ewald (Hudson) tissue forceps (12 cm), curved Kelly forceps, Meinhoeffer curette (1 mm), microdissecting forceps (4 inch serrated, full covered extra delicate), Maloney forceps with

serrated tip, auto clips (9 mm), auto clip remover (9 mm), and dissecting probe (9 mm).

A standard harness is employed to hold the cannula immobilized at the neck of the rats, and the cannula is connected to a single channel swivel for infusion of solution into the intestine.

The mesenteric lymphatic cannula consists of medical grade Silastic® tubing (0.020″ i.d. × 0.037″ o.d., Dow Corning Corporation, Midland, MI). The intestinal cannula consists of polyethylene tubing (PE10 and PE20) connected to Silastic® tubing (0.012″ i.d. × 0.025″ o.d.), and the jugular cannula consists of polyethylene tubing (PE20) connected to Silastic® tubing (0.020″ i.d. × 0.037″ o.d.). Syringe infusion pumps are used to control the rate of duodenal infusion. The infusion solution consists of normal saline USP supplemented with 0.09% potassium chloride. Standard 3-0 coated Vicryl® dissolvable sutures are used.

Serum and lymph are typically assayed for triglyceride, cholesterol, albumin, and protein using standard diagnostic assays for serum. Triglycerides may be measured using the glycerol dehydrogenase method[39] where the endpoint is based on NADH generated by glycerol-3-phosphate dehydrogenase. A standard curve is generated from serial dilutions of lymph using saline as diluent. Cholesterol may be analyzed using an enzymatic procedure[40] using a chromogen generated from peroxide produced by the oxidation of cholesterol. Albumin can be measured by the method of Doumas et al.[41] using the bromcresol green colored complex formed with albumin. Total protein may be measured using the modified biuret method[42] where the protein is complexed with cupric ions.

2. Jugular Vein Cannulation

Male Sprague Dawley rats weighing between 275 and 300 g are tranquilized with 50% CO_2/air for 1 min, followed by inhalation anesthesia with Metofane® (methoxyflurane, Pitman-Moore Inc., NJ).

The method for cannulating the jugular vein of rats was described by Weeks and Davis.[43] The dorsal area of the neck and the area between the ears on the back of the head is shaved and cleaned with sterile gauze and a mixture of zephiran solution and 95% alcohol (1:1). A spot is marked on the back of the head midway between the ears using a felt marking pen to indicate the exit point of the cannula. An incision about one inch long is made over the jugular vein and the muscle is dissected away using a probe and/or the pointed end of the surgical scissors. A cannula attached to a trocar is pulled by short jerking motions through the tissue just below the skin from the posterior of the incision down the midline, following the rib cage around the right forearm, and continuing to the back of the neck where it exits at the premarked location between the ears. The trocar is then removed and the cannula is attached to a 1 ml syringe filled with normal saline solution and a wound clip is applied to anchor the two knobs of the cannula in place between the ears. The 3-0 silk sutures which are attached to the cannula are used to secure the cannula to the base of the

sternomastoid muscle in the neck. Care is taken not to over-tighten the silk suture since muscle necrosis could occur.

A pocket is formed under the salivary gland with the curved end of a hemostat. The pocket is used to hold the loop of the silicone rubber cannula and to allow movement of the animal without disrupting the position of the cannula in the vein. With the aid of a glass probe and a microdissecting curette (1 mm tip), the vein is exposed by dissection. A cotton "Q Tip" bud is used to remove connective tissue from the vein. After isolating the vein, a probe is placed underneath it, and if the vein is constricted, pressure is placed on the right side of the heart, causing the vein to become filled with blood.

A 20 ga. needle is then used to puncture the wall of the vein. With the aid of microforceps, the tip of the cannula is passed under the cannula inserter and into the vein. The silicone rubber portion of the cannula is easily worked down the vein toward the heart. If there is difficulty in inserting the cannula (i.e., the cannula stops or bounces back after pushing it in) then it may have gone into one of the branching veins. In such cases, the cannula is pulled part of the way out and is then reinserted until it goes in easily and completely to the preset anchor point made of heat-shrinkable tubing. For rats weighing 275 to 300 g, this point is 32 to 33 mm from the tip of the cannula. The cannula is checked for blood withdrawal by pulling back on the 1 ml syringe. If the cannula is installed correctly, the tip of the silicone tubing should be in the right atrium of the heart and blood will be easily withdrawn. One drop of cyanoacrylate glue is placed near the entry point of the cannula into the vein, followed by a drop of saline to polymerize the glue.

The cannula is anchored in the muscle next to the point where it enters the jugular vein by tying the silk suture attached to the heat-shrinkable tubing to the muscle. The cannula loop is placed in the preformed pocket under the salivary gland and the wound is closed with several small drops of cyanoacrylate adhesive. Adhesive is first placed underneath the subcutaneous tissue along the side of the incision and the tissue is stretched over the cannula. This is done to prevent the tubing from rubbing directly on the skin. The skin is closed by pinching and gluing. Finally, the wound area is flushed with saline solution to finish polymerizing the glue and a plug is placed in the exit of the cannula.

The animals are allowed to recover in cages with free access to food and water. Recovery time varies somewhat from animal to animal, but from monitoring body weight loss/gain the average recovery time is about 3 days. After this time the abdominal surgery is performed as described in the following section.

3. Duodenal and Mesenteric Lymphatic Duct Cannulation

After a minimum recovery period of three days following jugular vein cannulation, the rats are fasted overnight and allowed free access to water. The rat mesenteric lymphatics are cannulated according to the general methods previously described.[12,13] One hour prior to surgery, 1 ml of corn oil is given

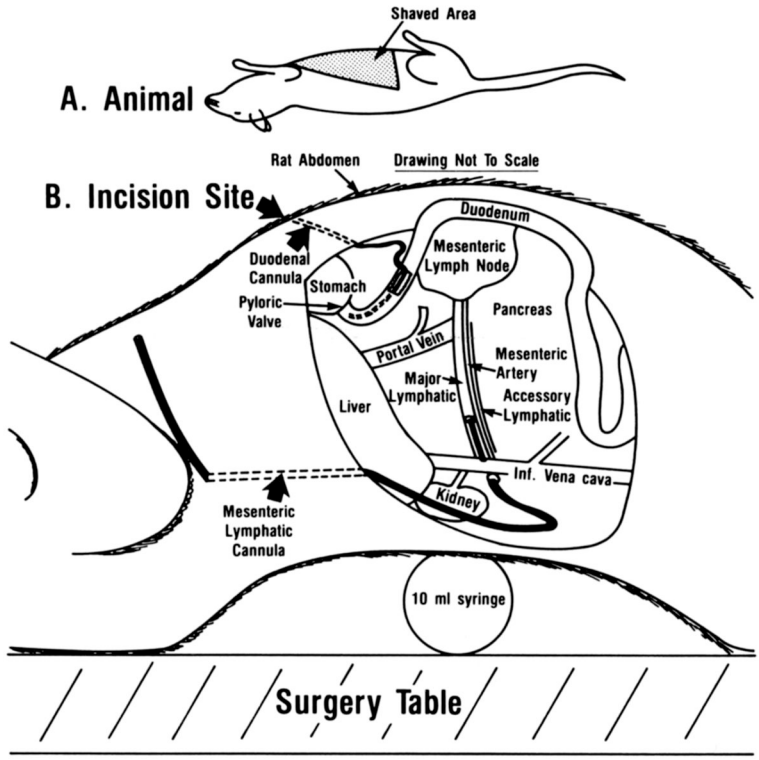


FIGURE 2. Detailed placement of (A) incision, and (B) placement of mesenteric lymphatic and duodenal cannulas. This drawing has been expanded to show detail. Cannulas are drawn in black and the dotted lines show their placement in the tissue.

orally to help visualize the lymphatics. The rats are tranquilized with a 50% mixture of CO_2 and air, and they are then anesthetized with Metofane®. A triangular area from under the right foreleg to the flank and to the midline is shaved and cleaned with alcohol. According to the method of Warshaw,[12] and as depicted in Figure 2, a right subcostal incision is made extending to the flank beginning with the tip of the sternum. Initially, a small 2-cm incision is made through the abdominal muscle to allow placement of the duodenal cannula without total disruption of the intestines. The cannula is placed on the end of a trocar and is run diagonally under the skin to the back of the neck near the exit of the jugular cannula. A wound clip is used to hold the cannula in place. After removal of the trocar, a saline line is connected to fill the cannula and to introduce a saline load prior to closure of the incision. The end of the cannula is kept outside the peritoneal cavity using a small bulldog clamp. The remaining muscle underneath the skin incision is cut, exposing the lower portion of the liver, duodenum, and the cecum. A section of 17 ga. needle tubing is pushed

through the muscle and under the skin where it exits under the right foreleg, parallel to the midline. The mesenteric duct cannula (silicone rubber tubing, one end has a 45° bevel, 200 mm, 0.02″ i.d. × 0.037″ o.d.) is pushed through the tubing, and then the needle tubing is removed. A short piece of metal tubing (1 cm, 20 ga.) is threaded over the cannula at the exit point to secure the cannula to the harness.

The animal is bridged over a 10 ml syringe for better visualization of the mesenteric lymphatics. The duodenum is pulled out from under the liver and is placed carefully over the midline to expose the mesenteric lymph duct. A towel forceps is placed around the duodenum and it is carefully clamped to avoid piercing the pancreas while holding the duct taut. A 4 cm × 4 cm piece of saline soaked gauze is placed over the area to keep the intestine moist. The vena cava is separated from underlying tissue by passing a glass probe underneath and following with microforceps. The beveled end of the cannula can then be threaded under the vena cava such that the cannula follows the mesenteric duct. A thin clear membrane covers the duct which must be removed using a very fine probe. If this membrane is not removed, a puncture can still be made in the duct but the cannula tends to go between the membrane and the duct. If this happens, it can be difficult to distinguish between a cannula that is properly placed but occluded, and one that is improperly placed. The cannula is then connected to a syringe filled with heparin (100 units/ml). A cannula inserter, made from a 20 ga. needle, is used to puncture the duct near the vena cava above any accessory lymphatics and a few millimeters of the cannula (bevel up) is inserted into the lymphatic duct. The mesenteric duct is fragile and, after the corn oil meal, it is distended and under slight pressure. If the cannula is not inserted correctly the first time, the duct will become deflated. Magnifying glasses will be required to visualize the hole and the small cavity will immediately fill with white lymph. If the puncture cannot be found or if the duct is torn, a second attempt can be made closer to the pancreas since lymph flows away from the intestine. The cannula is designed to collect all of the lymph so that the damaged area below the cannula will not cause loss of fluid. If small accessory lymphatic vessels are present adjacent to the mesenteric artery, these are then intentionally disrupted and sealed with a drop of cyanoacrylate adhesive to maximize collection of mesenteric lymph via the implanted cannula.

After removal of the inserter, one drop of cyanoacrylate adhesive is placed at the junction of the duct and cannula. To minimize intestinal or liver adhesion, several drops of saline are placed on the glued area which is allowed to stand for approximately 30 seconds to harden the adhesive. An additional drop of adhesive is placed on the cannula on both sides of the inferior vena cava to firmly secure the cannula to the vein, thereby enabling free movement of the rat without the risk of disrupting the cannula.

After the mesenteric lymphatic duct is cannulated, a duodenal cannula is inserted at a site approximately 1 cm from the pyloric valve. A bent 23 ga.

needle is used to puncture the intestine in an area avoiding the blood supply. The tip of the cannula is then inserted, with the aid of the attached bulldog clamp, to within a few millimeters of the pylorus. Cyanoacrylate glue is used to hold the cannula in place at the point of entry. To secure the cannula in place, an additional 0.5 cm of the cannula is glued along the side of the duodenum. Saline is used to polymerize excess glue, and the peritoneal cavity is shaken gently to allow the intestine to move back into place. The animal is given a 0.5 ml load of normal saline into the intestines to boost lymphatic flow and to discourage clotting of the lymph in the cannula.

The incision in the muscle is closed with absorbable sutures (3-0 coated Vicryl®) using a continuous over and over suture. The skin is closed using 9 mm wound clips dispensed by an autoclip. A plug is placed in the intestinal cannula exit if fluid is to be infused into the jugular vein.

The animals are placed in a saddle/swivel leash arrangement and the infusion line is connected to the duodenal or jugular cannula. The saddle strap is placed tight enough to keep the animal from pulling out, but not tight enough to obstruct lymph flow. The lymphatic cannula is secured to the strap using a 9 mm wound clip.

4. Lymph Collection and Blood Sampling

Animals are housed individually in wire bottom cages with free movement about the cage during the recovery period following the abdominal surgery. Mesenteric lymph is allowed to flow freely during the recovery period while a saline solution (0.9% NaCl containing 0.09% KCl) is infused continuously into either the jugular vein or the duodenal cannula at a rate of 2.2 ml/min. The animals are allowed water and solid food *ad libitum* for the first 24 hr following surgery but are denied food 24 hr prior to dosing and/or sample collection.

After allowing 48 hr for recovery from surgery, the rats are placed in a restraining cage. Dosing and blood sampling of the animal can be accomplished by temporarily stopping the saline infusion and administering the test material or taking blood samples. Collection of the lymph and/or blood via the jugular or lymph cannula allows for monitoring the delivery of the test compound.

Upon infusion of saline into the duodenum at a flow rate of 2.2 ml/hr, the average flow rate of lymph was 1.60 ± 0.4 ml/hr and this was similar to the value obtained using jugular infusion (1.9 ± 0.7 ml/hr) (Morozowich et al., unpublished results). Infusion of normal saline into the duodenum gave an increase in lymph flow from 1.6 to 6.6 ml/hr upon raising the saline infusion rate from 2.2 to 11.5 ml/hr (Figure 3). The slope of the linear relationship indicates that the lymph flow rate increases by about a factor of 0.5-times the saline infusion rate. A similar linear relationship between saline infusion rate and lymph flow has been reported.[13] Duodenal saline infusion flow rates as high as 7.3 ml/hr were tolerated by the rats as judged by normal grooming and eating behavior and the normal appearance of stools. Higher infusion rates resulted in a lethargic appearance in the animals.

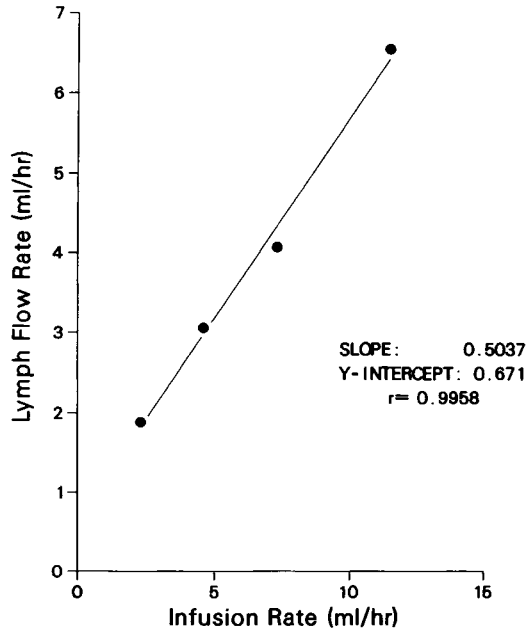


FIGURE 3. A plot showing the relationship between mesenteric lymph flow rate and rate of intestinal infusion of normal saline. Lymph flow will eventually stop and the cannula will become plugged if continuous saline infusion is not used.

5. Assessment of the Conscious Triple-Cannulated Rat Model for Lymphatic Delivery Studies

The protocol described conveniently allows one lymphatic delivery study to be performed per week with three to four animals by one operator. The described model requires patience and surgical practice to obtain a survival rate of approximately 80%, although it is reasonable to expect that a skilled operator will achieve success rates of greater than 80%.

The rats appear to tolerate jugular surgery better than the abdominal surgery as determined by weight loss. For example, the observed body weight loss in rats (initial weight 275 to 300 g) after jugular surgery was about 3%, whereas the weight loss after abdominal surgery was approximately 12%. The weight loss after jugular surgery was readily regained, although this was not the case after abdominal surgery. The rats appeared to be in good health based on their appearance, eating habits, and stool formation. Near normal grooming behavior is seen on the first day after surgery and the animal presents a healthy appearance (Morozowich et al., unpublished results). The animals that are allowed to recover for 5 days after surgery typically have bitten off their lymphatic cannulas along with some of the abdominal wound clips.

Insertion of the duodenal cannula is readily accomplished, although animal survival depends on the magnitude of the induced surgical stress and associated trauma. Consequently, the time of the operation should be kept to

a minimum and the time of exposure of the intestines to air should be minimized in order to prevent ileus formation.

The cannulation of the mesenteric lymphatic duct is the most difficult of the three cannulations due to its fragility and the difficulty in isolating the lymphatic duct. The administration of a lipid prior to the surgery is useful in locating the lymphatic duct since this gives the duct a whitish appearance and it is easily located below the portal vein. Bridging the animal by placing a 10 ml syringe barrel under the back of the animal is helpful in determing the location, and subsequent cannulation of the lymphatics.

A saline (containing 0.09% KCl) infusion rate of 2.2 ml/hr is preferred for animal hydration since this flow rate is well tolerated for both jugular and duodenal infusion of saline. Faster flow rates in the jugular vein result in increased tendency for pleural congestion.

The triglyceride output during the first 3 hr after placement in the restraining cage is often erratic and may be due to the initial stress associated with caging. For this reason, it is proposed that lymphatic absorption studies should be initiated with dosing 3 hr after placement of the rat in the restraining cage.

Baseline blood values for lipid and protein content were obtained in fasted animals. The blood values for total protein and albumin decreased slightly after lymph flowed for 3 days from an initial value 5.5 ± 0.5 to 4.4 ± 0.6 g/dl for total protein and from an initial value of 2.6 ± 0.3 to 2.0 ± 0.2 g/dl for albumin. The initial values are consistent with those reported in the literature, namely, 5.8 g/dl for total protein and 3.2 g/dl for albumin in the normal rat.[44] The triglyceride plasma level (57 ± 10 mg/dl) decreased slightly after cannulation (44 ± 12 mg/dl) and the cholesterol levels increased marginally from 56 ± 8 mg/dl to 68 ± 17 mg/dl after cannulation. Overall, these data indicate that there was not a major change in the blood chemistry profile of the rats subjected to these cannulation procedures.

The triglyceride content of the mesenteric lymphatics in the fasted rat, although variable, is generally between 200 and 400 mg/dl after saline hydration via either the duodenal or jugular cannulas. Therefore, the resulting hourly fasted triglyceride output calculated from the lymph flow rate is 5.3 to 5.6 mg/hr, which is in good agreement with reported values (2.4 to 5 mg/hr).[35]

It is important to co-administer a triglyceride emulsion when conducting lymphatic delivery studies to provide the basis for lipoprotein formation. Triolein emulsions and Intralipid® are two of the commonly employed emulsions. Bolus injections of 0.5 to 1 ml of lipid are acceptable, or alternatively, lipid can be infused over a 5-hr period at a rate of 50 to 100 mg/hr. The recovery of duodenally infused lipid in the intestinal lymph typically ranges between 50 to 90% of the administered dose. Figure 4 indicates that triglyceride recovery amounted to approximately 60 to 80 mg/hr after 3 hr of infusion when a triolein emulsion (31.3 mg/ml) was administered by constant infusion at 2.2 ml/min. These recovery rates are consistent with the data of Mansbach and Arnold[22] using similar infusion rates of triglyceride.

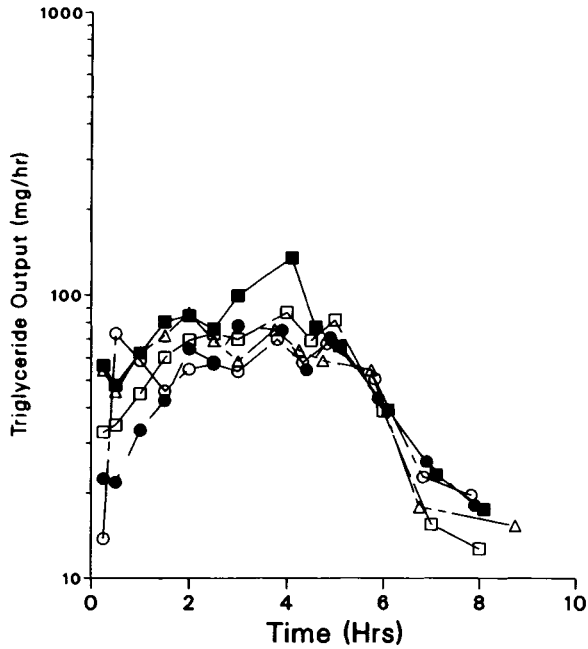
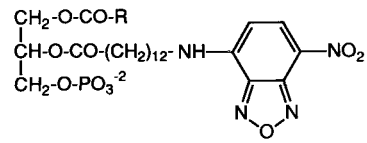


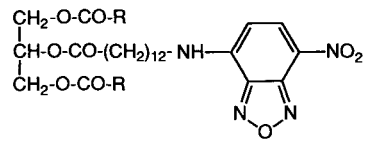
FIGURE 4. Lymph triglyceride output (mg/hr) as a function of time in rats (n = 5) infused for 5 hr with a triolein emulsion (31.3 mg/ml) at a rate of 2.2 ml/hr. The different symbols represent results from individual rats.

The applicability of the conscious rat lymphatic delivery model for assessing intestinal lymphatic absorption was validated by a study of the lymphatic transport of intestinally administered 1,1-bis(4-chlorophenyl)-2-trichloroethane (DDT) and p-aminobenzoic acid (PABA). DDT is a commonly used highly lipophilic compound which is selectively transported via the intestinal lymphatics, and PABA is primarily absorbed via the portal blood due to its hydrophilic character. Both compounds were administered via a duodenal cannula in the cannulated rat model and the drug content in the mesenteric lymph was determined by HPLC.

The amount of PABA delivered into the lymph was found to be less than 3% of the administerd dose, which is consistent with absorption via the portal blood rather than lymphatic transport. The amount of DDT transported in the intestinal lymph ranged between 60 and 80% (n = 4) which compares favorably with that reported by Pocock and Vost[45] who reported 60% transport of the administered DDT dose in a conscious rat model. This value is considerably higher than the levels of transport (approximately 30 to 40% of the administered dose) reported in a similarly cannulated rat model which was anesthetized for the duration of the experiment.[20] It is likely that the differences in transport between the conscious and anesthetized models reflect the disturbance of the normal physiological processes due to the anesthetic. It is anticipated that if an



C12-NBD-PA



C12-NBD-TG

FIGURE 5. Chemical structures of C12-NBD-PA and C12-NBD-TG.

anesthetized rat model is utilized for estimating lymphatic transport, then the trends in the transport data are the important experimental outcome and not the absolute extent of lymphatic transport.

The utility of the triple-cannulated conscious rat model was further assessed by studying the lymphatic transport of a highly fluorescent probe which has been used to study the pathways of lipid transport in cultured cell systems.[46] The compound C12-NBD-PA (1-acyl-2-{12-{(7-nitro-2-1-3-benzoxadiazol-4-yl)-amino} dodecanoyl}phosphatidic acid) has been shown to undergo uptake in cell culture upon membrane cleavage of the phosphate ester producing the corresponding diglyceride. The diglyceride then undergoes transbilayer internalization followed by reacylation giving a fluorescent triglyceride deposit with the nitrobenzodioxazole (NBD) label remaining at position 2 to form C12-NBD-TG (1-acyl-2-{12-{(7-nitro-2-1-3-benzoxa-diazol-4-yl)-amino}-dodecanoyl}-3-palmitoyl-glycerol).[45] Figure 5 presents the chemical stuctures of C12-NBD-PA and C12-NBD-TG.

In an attempt to determine whether similar uptake transformations occur *in vivo* in the triple-cannulated rat model thereby leading to lymphatic delivery, 0.5 mg of C12-NBD-PA (Sigma Chemical Co.) was dispersed in 1 ml of a triolein emulsion (50 mg/ml) and made isotonic with glycerin (300 mOsmoles). As described, the rats were allowed to recover from the above surgeries with continuous administration of normal saline into the duodenum at a rate of 2.2 ml/hr, and free access to food and water was allowed on the first post-surgery day. The animals were fasted overnight prior to the second day after surgery, and 5 min prior to administration of 1 ml of the C12-NBD-PA formulation, a triolein emulsion was infused at a rate of 2.2 ml/hr. The C12-NBD-PA formulation was administered into the saline infusion line connected to the duodenal cannula with temporary interruption of the triolein infusion. After sample

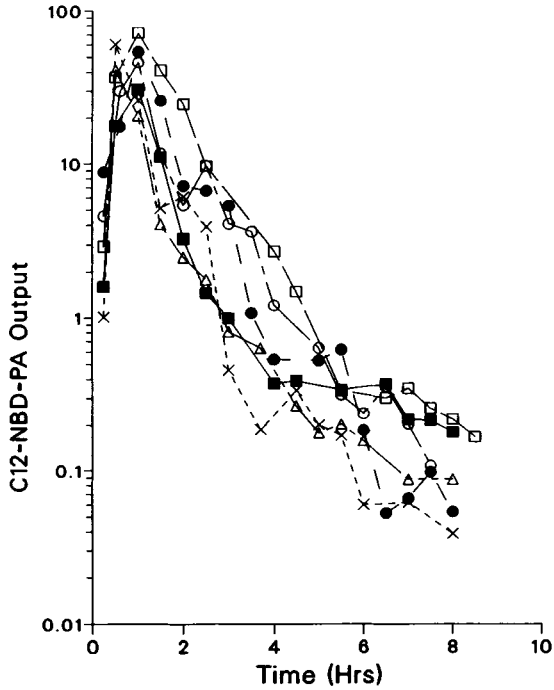


FIGURE 6. A plot showing the amount of C12-NBD-PA (μg/hr) obtained in the mesenteric lymph of rats (n = 6) after administration of a 0.5 mg bolus dose. See text for details of the administration. The different symbols represent results from individual rats.

administration, the triolein emulsion was infused at 2.2 ml/hr for a total period of 4 hr. Mesenteric lymph samples were collected in 30-min periods (in heparinized vials) for a period of 12 hr with a final sample taken at 24 hr. The samples were assayed for NBD content upon treatment with 0.2 ml of 0.05 *N* HCl and subsequent extraction with 5 ml of chloroform/methanol (9:1) followed by determination of total fluorescence intensity using an excitation wavelength of 480 nm and an emission wavelength of 535 nm.

The typical concentration-time profile for lymphatic delivery of the NBD fluorophore is shown in Figure 6 from which a cumulative delivery plot can be derived (Figure 7). In this case the species in the lymph was the corresponding triglyceride C12-NBD-TG which is formed after cleavage of the phosphate moiety in C12-NBD-PA and subsequent reacylation to produce C12-NBD-TG. This profile is similar to that reported in cell culture.[46]

D. OTHER INTESTINAL LYMPHATIC DELIVERY MODELS

1. Dog Lymphatic Delivery Model

The procedure that we use for creating a lymph fistula in the dog is a modification of that previously described by Rajpal and Kirkpatrick.[47] With

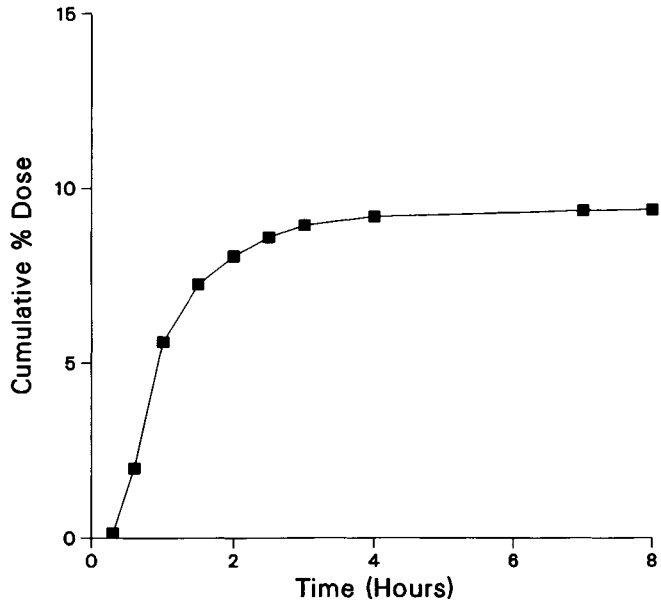


FIGURE 7. Mean cumulative transport (% administered dose, n = 5) of C12-NBD-PA in the mesenteric lymph of rats after administration of a 0.5 mg dose of C12-NBD-PA.

this approach the thoracic duct is not actually cannulated, but rather, the external jugular vein is ligated above and below the point at which it is joined by the thoracic duct and a cannula is sutured into that section of the vein. The lymph then flows into the cannula via the pouch that is created by ligation of the vein. The advantage of this procedure is that one is able to obtain the lymph without performing a thoracotomy.

Approximately 4 hr before surgery, the dog is fed one-half can of dog food (meat). Just prior to surgery, the dog is lightly anesthetized with phenobarbital and his neck shaved and washed with Microbicidal Betadine Surgical Scrub (Purdue Frederick Company, Norwalk, CT). The trachea is intubated and the anesthesia maintained with a mixture of halothane and oxygen. The animal is positioned on the table on his back, with the head turned to the right so as to expose the left side of the neck. The left external jugular vein is then exposed surgically and ligated 1 cm inferior to its junction with the omobrachial vein. The superficial cervical, cephalic, and subclavian veins are ligated at their junctions with the external jugular, and the internal jugular is ligated at its junction with the brachiocephalic vein. The brachiocephalic vein is ligated just inferior to that junction. The isolated portion of the external jugular is then drained of blood and opened longitudinally. A valve is usually present over or just cranial to the point at which the thoracic duct enters the vein, and that valve is gently removed. The preparation is then examined to make certain that lymph is still flowing and that it is free of blood. If there is significant blood

in the lymph, it can cause clotting and interrupt the lymph flow. A large cannula, previously coated with TDMAC-heparin (Polysciences, Inc., Warrington, PA) is sutured into the isolated portion of the external jugular just cranial to the orifice of the thoracic duct, and the entire incision is closed. The external portion of the cannula is then connected to a 250 or 500 ml blood donor bag containing ethylenediaminetetraacetic acid (EDTA) and preservatives (see Section II B). A surgical stocking is then placed over the neck and thorax, with holes for the front legs, and the lymph collection bag is secured to the inside of the stocking. The overall appearance is that of a dog wearing a turtle-neck sweater with the lymph bag attached to the inside of the sweater.

The dogs are allowed to recover from the surgery (usually 24 hr is sufficient) and are then administered the experimental meal/formulation containing the compound of interest. The lymph collection bags are changed as necessary, and the lymph flow from a 20 kg dog fed a large meal can be greater than 100 ml/hr.

2. Pig Lymphatic Delivery Model

A lymphatic transport model has been recently described which employs an anesthetized pig.[48] The model enabled the simultaneous sampling of mesenteric lymph, hepatic portal blood, and systemic blood. Although the goal of the reported study was to determine the absorption pathways of an administered compound (propranolol) in the absence of co-administered lipid, it should be possible to adapt the experimental system to the study of lipoprotein-based lymphatic transport. Cannulation of the mesenteric lymphatics was facilitated by the prior administration of a lipophilic dye (sudan black) dissolved in lipid on the evening prior to surgery.

II. ANALYSES OF LYMPH

A. LYMPH LIPOPROTEINS VS. PLASMA LIPOPROTEINS

Lipoproteins synthesized in the intestinal mucosal cells are transported to the lacteals of the intestinal villi and shortly thereafter mix with lymph flowing from the periphery. Post-nodal peripheral lymph has invariably been contaminated to some degree with plasma, so it is virtually impossible to obtain nascent intestinal lipoproteins by lymph duct cannulation which are not contaminated to some extent with plasma lipoproteins. In addition, essentially all of the known plasma apoproteins are present in peripheral lymph, and those apoproteins quickly adsorb to, or exchange with, the apoproteins on the surface of nascent intestinal lipoproteins. Therefore, it is also extremely difficult to obtain nascent intestinal lipoproteins that contain only proteins synthesized in the intestine. Consequently, it must be realized that regardless of the method employed for the isolation of intestinal lipoproteins, there will be some degree of contamination by plasma lipoproteins and apoproteins. Nonetheless, a great deal of information can be obtained regarding the absorption and transport of li-

pophilic compounds by lymph duct cannulation, and the methods of isolation and characterization of the lipoproteins in the lymph are reasonably simple and straightforward.

B. PROCESSING AND STORAGE OF LYMPH FOR ANALYSES

Chyle is collected from a mesenteric duct cannula as a function of time by using a fraction collector. The lymph is dripped into chilled tubes or vials containing several different additives as discussed below. In general, lymph should be handled as is recommended for plasma[49] and although lymph can be stored at 4°C for several days, it is best to use it as soon as possible. Although it is possible to freeze lymph if it cannot be used within 2 to 3 days, this practice should be avoided since lipoproteins may be destroyed and low temperatures may alter the centrifugal properties of the respective lipoprotein fractions.[50]

An anticoagulant such as EDTA (0.01 to 0.05% w/v) is added to each collection tube. The EDTA will also act as a protease inhibitor, especially of metalloproteases, by chelating metal cofactors. Chelation will also prevent heavy metal catalyzed oxidative degradation of lipoproteins.[51] It is possible to substitute sodium heparin at 100 units/ml for EDTA; however, the additional metal chelation benefits of EDTA would be lost. Since the different enzymes present in lymph may remain active even at low temperatures, several different inhibitors are added to each sample of collected lymph. A cocktail of protease inhibitors with a variety of specificities can be added and we routinely use phenylmethylsulfonylfluoride (PMSF) at 2 m*M*, aprotinin at 20 trypsin inhibiting units per ml, and leupeptin at 1 μg/ml. In addition to protease inhibitors, inhibitors of lipases and lipid transferases can be added as is typically done for blood plasma. For example, 1 m*M* Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) inhibits lecithin:cholesterol acyltransferase (LCAT) which esterifies free cholesterol to cholesteryl esters. However, this may not be necessary as peripheral lymph has LCAT levels that are 100-fold lower than for plasma.[52] The addition of thimerosal, which has bacteristatic properties, may also inhibit lipoprotein lipase.[53]

It is wise to add a bacteriostatic to any biological fluid provided that its addition does not interfere with subsequent analyses. As mentioned, we use thimerosal due to its added protection against lipase. Sodium azide can also be used, although it has been suggested that azide may stimulate oxidative reactions,[54] and under these circumstances we suggest the addition of EDTA. The oxidation of lipoproteins, lipids, or drugs within lymph can be quelled by low temperature and the addition of glutathione at 0.02% (w/v).[55]

Prior to analysis and storage, chilled lymph is subjected to low speed centrifugation at 500 x*g* for 10 min to remove debris and whole cells (e.g., lymphocytes). If lymph is stored for several hours at 4°C, the fibrin will precipitate and an additional centrifugation is required.

C. ISOLATION OF LIPOPROTEIN FRACTIONS FROM LYMPH

1. Fractionation of Intestinal Lipoproteins by Density

Intestinal lipoproteins are large lipid-rich particles with a density substantially less than the solution density of lymph or plasma. The largest of the lipoproteins have a density of 0.94 g/ml or less, whereas the background salt density of plasma or lymph is 1.0063 g/ml.[56] Therefore, with time the majority of those lipoproteins will float to the surface of that solution in the same way that cream floats to the surface of whole milk. By centrifuging the lymph, one can substantially speed that process and also partially separate the lymph lipoproteins into various size or density fractions. However, whereas plasma lipoproteins can for the most part be separated into distinct metabolic entities by ultracentrifugation,[57] this is probably not the case for intestinal lipoproteins. This is because the size or density distribution of intestinal lipoproteins appears to be a continuum[58] and fractionation based on size or density is, to a large extent, arbitrary. Nonetheless, most investigators find it convenient to separate lymph lipoproteins into four fractions defined by their density or Svedberg flotation rates.[59] These fractions are: $S_f > 400$ (chylomicrons), $S_f = 20 - 400$ (very low density lipoproteins or VLDL), $S_f = 0 - 20$ (low density lipoproteins or LDL, and high density lipoproteins or HDL). The $S_f = 0 - 20$ fraction is sometimes further subdivided into the $S_f = 12 - 20$ and $S_f = 0 - 12$ fractions.

There are three types of ultracentrifuge rotors that can be used for lipoprotein separations: fixed angle rotors, vertical rotors, and swinging bucket rotors. The choice of rotor will depend upon the number and volume of the samples to be analyzed. We prefer the swinging bucket rotors for the simple reason that the tube and its contents are always oriented in the direction of the force field (i.e., the bucket swings upward during acceleration and downward during deceleration and therefore the wall effects present in fixed angle or vertical rotors are greatly reduced). The following method is for separation of lymph lipoproteins in the Beckman SW-40Ti rotor, but can be easily adapted for use with smaller or larger swinging bucket rotors or fixed angle rotors. We have had no experience fractionating intestinal lipoproteins in a vertical rotor and the reader is referred to an excellent review of that method by Chung and coworkers.[60]

The volume of the tubes used in the SW-40Ti rotor is approximately 14 ml. However, sample size should not exceed 10 ml so that sufficient room is left at the top of the tube for an overlay solution through which the lipoproteins can wash as they move to the top of the tube. The minimum sample size is dependent to a large extent on the sensitivity of the methods used to detect the compound of interest. However, if the sample size is less than 0.5 ml, we recommend using one of the smaller swinging bucket rotors (e.g., SW-50.1, SW-55Ti, or SW-60Ti).

We find it preferable to layer the lymph sample under a stock NaCl solution (prepared as described below), rather than trying to layer the NaCl

solution over the lymph. Depending on the volume of lymph to be centrifuged, 4 ml or more of the NaCl solution are added to the ultracentrifuge tube, and the tube placed in what is called a "full-view test tube rack" which is designed for 10 to 13 mm (i.d.) tubes. The racks consist of rows of pins, between which the ultracentrifuge tube can be wedged, and are preferred because the ultracentrifuge tube can be adjusted up or down in the rack. A 12 ml syringe, from which the plunger has been removed and which has been fitted with a 21 ga., 3.5-inch needle, is suspended above the centrifuge tube with the needle reaching down into the tube. The tube is then adjusted in the rack so that the tip of the needle just touches the bottom of the tube beneath the NaCl solution. The lymph is then placed in the syringe and allowed to flow by gravity into the tube. Gentle pressure using either the thumb or the syringe plunger is sometimes required to start the flow, but thereafter it should proceed automatically with the lymph forming a distinct layer under the NaCl solution. Once all of the lymph has entered the tube, the syringe and needle are removed and the height of fluid in the tube is adjusted by gentle addition or removal of NaCl solution from the top until the top of the meniscus (where it touches the wall of the tube) is 3 mm from the top of the tube. For example, if the volume of the lymph sample was 8 ml and this was layered under 6 ml of the NaCl solution, then only minimal adjustment of the fluid height would be required. When odd numbers of samples are being analyzed, balance tubes, to be hung opposite the sample tube on the rotor, are prepared by simply filling the tubes with d = 1.006 g/ml NaCl solution.

Once all of the samples have been layered under the NaCl solution, the tubes are placed in the rotor buckets. It is vital for this and subsequent centrifugation that the inside of the bucket and the outside of the tubes be completely dry, as liquid trapped between the tube and the bucket can cause the tubes to collapse during the centrifugation. Also, it is important that the O-ring at the top of the bucket has a thin coat of vacuum grease, and we routinely remove the O-rings, clean them, and re-grease them before every use. Once the tubes are firmly seated in the buckets, the caps are installed and the tubes are carefully placed on the appropriate rotor hanger. We routinely hang all of the buckets on the rotor (even though some may be empty). The rotor and buckets are pre-cooled to 4°C prior to placing the sample tube in them. All subsequent centrifugation is carried out at that temperature.

For isolation of $S_f > 400$ lipoproteins, we centrifuge at 40,000 rpm for 1 hr and 20 min and use the brake to speed deceleration (the brake automatically disengages at approximately 500 rpm). The lipoproteins that have floated into the top 2 cm of the tube are collected by slicing the tube with a knife at the appropriate point. Tube slicers designed specifically for this purpose can be purchased, and the Beckman Tube Slicer (Beckman Instruments) is one such example. Once the knife passes through the tube, it acts as a barrier, which enables collection of the fraction in the top of the tube (above the knife blade) without disturbing the layer below the blade.

When the $S_f > 400$ fraction is floated from lymph collected postprandially, it often forms a thick, semi-solid layer at the top of the tube. Once the tube is sliced, the majority of that layer can be collected with a small spatula and transferred to another container. The top of the tube is left in place (in the slicer) and the remaining $S_f > 400$ lipoproteins are then collected by repeatedly washing that portion of the tube with the stock saline solution. Bubbles should be avoided when rinsing the lipoproteins from the side of the tube and the knife since air-water interfaces disrupt lipoproteins and can irreversibly denature them. For that reason, we prefer to gently rinse the tube and knife using a pasteur pipet, rather than using, for example, a needle and syringe. Once all the $S_f > 400$ lipoproteins have been transferred to the container, they are dispersed by gently rinsing them back and forth in a pasteur pipet until no clumps are visible. Again, it is important to avoid bubbles during that procedure.

The $S_f = 20 - 400$ lipoproteins are obtained by layering the $S_f < 400$ fraction (that remaining in the bottom of the tube after the top 2 cm have been removed) under 2.8 ml of the stock NaCl solution as described for the $S_f > 400$ fraction, and centrifuging those tubes at 40,000 rpm for 16 hr. Again, the top 2 cm of the tube are taken by tube slicing. To obtain the $S_f = 0 - 20$ lipoproteins, the $S_f < 20$ fraction is adjusted to d = 1.063 g/ml with solid KBr or NaBr as described below, placed in a clean ultracentrifuge tube and overlaid with d = 1.063 g/ml solution. We use a 22 ga., 1-inch needle attached to a 5 ml syringe for layering. As with under-layering, the final height of the solution in the tube is adjusted so that it is 3 mm from the top. Finally, the HDL are floated by adjusting the $S_f = 0 - 20$ infranatant to d = 1.21 g/ml with solid KBr and centrifuging those fractions at 40,000 rpm for 44 hr.

There are a variety of approaches that are currently used to adjust the density of the sample and prepare overlay solutions. We feel that the simplest and most precise method of adjusting the density of a given solution is by the addition of solid KBr or NaBr. The standard formula for calculation of the amount of salt to add to a sample of given volume is:

$$wt.\ (g)\ salt\ required = \frac{V_i\ (D_f - D_i)}{1 - (v)\ (D_f)} \quad (1)$$

where V_i is the corrected volume of the solution to be adjusted, D_f is the density to which the solution is to be adjusted, D_i is the initial density of the solution to be adjusted, and v is the volume (milliliters) of water displaced per gram of the salt to be added. The corrected volume refers to the volume of the sample after correction for the effect of solids other than salt (proteins and lipid) on that volume. The protein and lipid concentration of intestinal lymph varies but generally averages about 5% by volume. Therefore, we routinely multiply the actual sample volume by 0.95 to determine the corrected volume for calculation of the salt required.

TABLE 1
Amount of KBr Required for Adjustment of Density of Samples from D_i to D_f

Fraction	D_i (g/ml)	D_f (g/ml)	KBr (g)
$S_f > 20$	1.006	1.006	0
$S_f = 0 - 20$	1.006	1.063	0.0829
HDL	1.063	1.21	0.2334
Total Lipids	1.006	1.21	0.3239

The volume of water (milliliters) displaced by the salt, often referred to as the partial specific volume of the salt, is calculated from the Concentrative Properties of Aqueous Solutions: Conversion Tables, in the *Handbook of Chemistry and Physics*. To do so, one finds the D_f in the relative density column (some interpolation is usually required) and, reading across the page, determines the corresponding C_s and $(C_o - C_w)$ terms. The value of v is then calculated from the formula:

$$v = \frac{C_o - C_w}{0.998\ C_s} \tag{2}$$

A stock NaCl solution of d = 1.006 g/ml is prepared and used for the subsequent preparation of higher density salt solutions. The stock solution is prepared by mixing 11.4 g of NaCl and 1 ml of a 10% (w/v) EDTA solution brought to a final volume of 1 l with distilled water. The density of the resulting solution should be exactly 1.006 g/ml as determined by a commercial density meter or pycnometer, and adjusted with H_2O or concentrated KBr solution as necessary. The stock solution is stored at room temperature in a tightly sealed container. A solution of any density between 1.006 and 1.21 g/ml can be easily prepared by simply adding the appropriate amount of solid KBr to a given volume of the stock NaCl solution as determined from Eq.(1). The amount of KBr required per milliliter of solution for some of the standard density intervals is shown in Table 1.

Figure 8 presents the distribution of cholesterol, triglyceride, and radiolabeled retinol among the $S_f > 400$, $S_f = 20 - 400$ and $S_f < 20$ lipoprotein fractions of thoracic duct lymph from a dog using the described ultracentrifugation method.[61] Figure 9 presents the time dependent transport of a fluorescent analog of phosphatidic acid, C12-NBD-PA, from the intestinal lumen of rats. The distribution of the fluorescent analog in the different lipoprotein fractions which appeared within chyle over the 4-hr period post-dosing was as follows: 92.8 ± 1.2% (w/w) was associated with the $S_f > 20$ fractions as C12-NBD-TG, 76.1 ± 1.2% (w/w) with the $S_f > 400$ fraction, and 16.7 ± 0.3% with the $S_f = 20 - 400$ fraction.[62] These profiles describing the transport of radiolabeled retinol and a fluorescent lipid illustrate how the approach can be employed to follow the absorption and distribution of lipophilic compounds within the lymph.

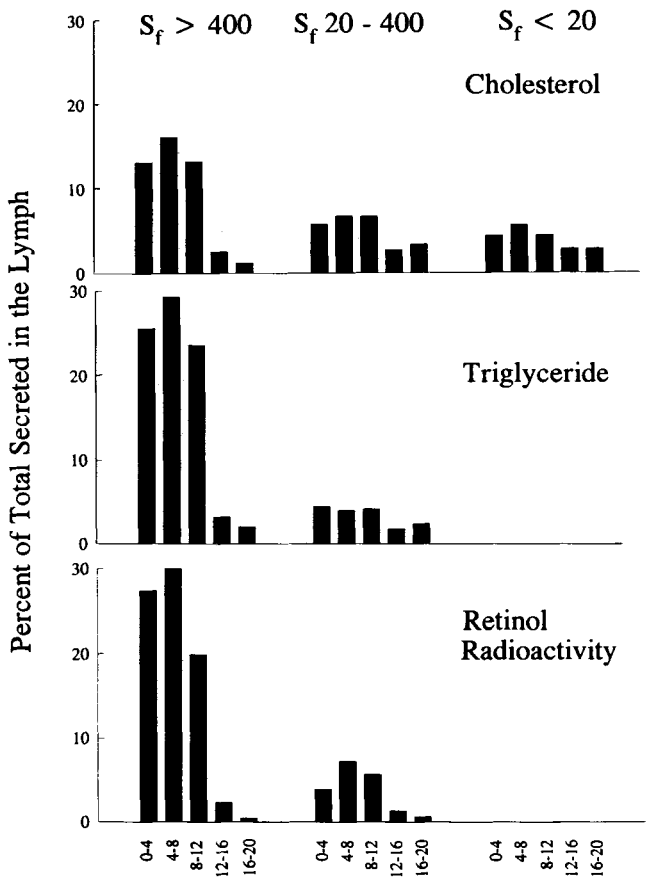


FIGURE 8. The distribution of cholesterol, triglyceride, and radioactive retinol among three density fractions of canine thoracic duct lymph. A lymph fistula was created as described and the dog allowed to recover for 24 hr. The dog was then fed a high fat meal containing radioactive retinol (at t = 0), and the lymph collected continously for 24 hr thereafter. The collection bag was changed every 4 hr and an aliquot of each collection bag taken for lipoprotein distribution analysis using the ultracentrifugation method described. Each bar represents the quantity of cholesterol, triglyceride, or radioactivity recovered in the 4 hr interval divided by the total quantity of that component recovered. Only trace amounts of triglyceride or retinol radioactivity were recovered in the $S_f = 0 - 20$ or HDL fractions (combined in the figure as $S_f < 20$). The $S_f < 20$ cholesterol was approximately equally divided between the $S_f = 0 - 20$ and HDL.

2. FRACTIONATION OF INTESTINAL LIPOPROTEINS BY COLUMN CHROMATOGRAPHY

Ultracentrifugation is the simplest and most rapid method for the fractionation of intestinal lipoproteins; however, it has the disadvantage that it may actually alter some of the lipoproteins (especially the HDL) by shearing apoproteins from the surface of the lipoproteins. Furthermore, both plasma and lymph contain very small lipid-poor lipoproteins which can only be isolated

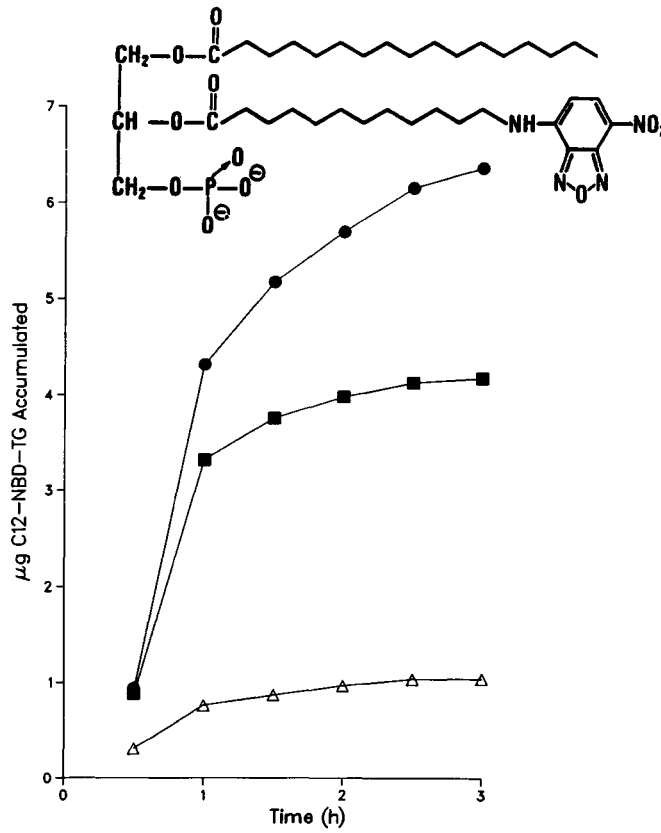


FIGURE 9. Cumulative appearance of a fluorescent analog of phosphatidic acid (C12-NBD-PA; structure shown) within intestinal lymph following perfusion of rat jejunum. Lymph was fractionated by density gradient centrifugation into a $S_f > 400$ fraction or chylomicrons (●) and a $S_f = 20 - 400$ fraction or very-low density lipoproteins (■). Fluorescence appearing within the lymph was identified as C12-NBD-triglyceride. The amount of fluorescence in the remaining portions of the gradient was measured (△).

using very high salt concentrations.[63,64] The effect of salt on lipoprotein integrity can be of concern especially when isolating the higher density lipoproteins.

Agarose column chromatography is an alternate method for fractionation of the intestinal lipoproteins which, although more labor intensive, avoids most of the problems associated with the ultracentrifugal technique. It is most useful for characterizing the size distribution of the $S_f < 400$ particles since $S_f > 400$ lipoproteins are excluded from the gel pores and appear in the void volume. For compounds which are distributed in the smaller intestinal lipoproteins (or in those instances when absorption of the compound is followed in fasted animals), this method can be especially useful since it

provides the investigator with the complete size spectrum of the lipoproteins with which the compound is associated, including lipoproteins as small as 40 to 50 kD.

There are a variety of commercially available agarose gels and columns which can be used for lipoprotein fractionation. We prefer Bio-Gel® A-15M (Bio-Rad Laboratories) for most lipoprotein separations as, in our hands, it appears to give better resolution than the other types of agarose we have tested (the reason for the differences among the commercially available sources of agarose is not clear). The column dimensions should be 2.5 (i.d.) × 100 cm and the flow rates should be 15 to 20 ml/hr. Columns of 1.5 cm (i.d.) can be used as they significantly reduce the degree to which the samples are diluted; however, wall effects are more evident and the resolution of the various fractions is significantly reduced. We routinely use phosphate buffered saline (pH 7.4) containing EDTA (0.01% w/v) and azide (0.02% w/v) as the column buffer. All separations are performed in the cold room so that temperature of the sample, column and fractions are maintained at 4°C.

The gel is equilibrated with the buffer and degassed overnight in the cold room prior to pouring the column. The gel is poured as a reasonably thick slurry. We usually remove all but 10 to 15% (v/v) of the liquid from the settled gel, and then adjust the volume by adding back buffer to the point that air no longer gets trapped in the slurry when it is mixed. A few milliliters of buffer are added to the column prior to pouring the gel, the stopcock is opened, and the gel is poured and allowed to settle with the buffer flowing. The time required for the gel to settle is generally about 4 to 5 hr. A flow adapter is fitted to the top of the column and the buffer is pumped at 20 ml/hr overnight and the flow adapter readjusted if necessary. When a new column is poured, we routinely pass the complete lipoprotein fraction (the $d < 1.21$ g/ml fraction described in Table 1) from 5 ml of human or 10 ml of Cynomolgus monkey plasma through it to evaluate its ability to resolve the lipoproteins. A typical elution pattern for the various lipoproteins is shown in Figure 10. A column is not considered acceptable unless the apo B-containing lipoproteins are completely separated (less than 2% overlap) from the HDL.

The volume of the lymph samples to be analyzed can range between a few hundred microliters to as much as 10 ml, with the separation being limited only by the sensitivity of the detection method. If very small samples (< 1 ml of whole lymph) are to be analyzed, it is suggested that the buffer contain bovine serum albumin at a concentration of 1 mg/ml to prevent interaction of the lymph apoproteins with the gel. Prior to loading lymph which is rich in intestinal lipoproteins, the $S_f > 400$ lipoproteins should be removed by ultracentrifugation as described above. The $S_f < 400$ lipoproteins (that material remaining in the ultracentrifuge tube) are then passed through a 0.45 μm filter and loaded onto the column. We prefer loading samples by simply transferring the inlet tube from the buffer reservoir to the sample container, and pumping the sample onto the column at the buffer flow rate

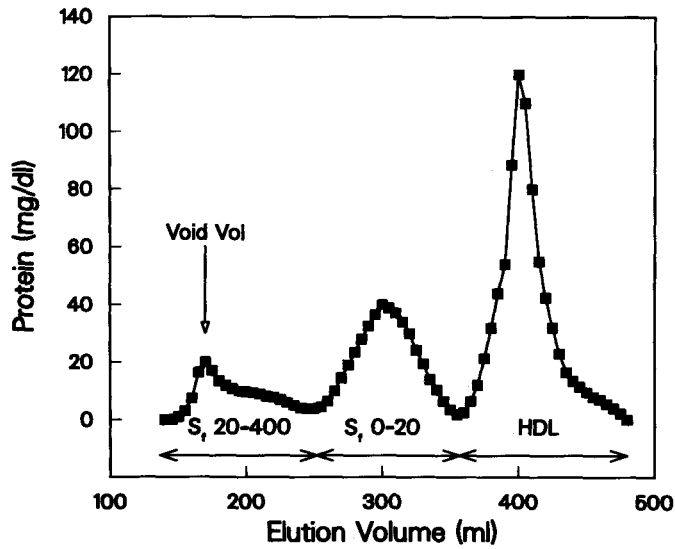


FIGURE 10. Elution profile of plasma lipoproteins separated by agarose (4%) column chromatography. KBr (3.27 g) was added to 10 ml of plasma from a Cynomolgus monkey and that plasma then centrifuged at 40,000 rpm for 44 hr using the SW-40 rotor. The tube was sliced as described and the lipoprotein fraction loaded onto the agarose column. This elution profile is typical of what should be obtained if the column is behaving properly. Note that in most instances *uncentrifuged* plasma or lymph from which the $S_f > 400$ fraction has been removed, is loaded onto the column and the elution profile of the compound of interest followed.

(15 to 20 ml/hr). The size of the fractions collected can vary, but 5 to 7 ml fractions give good resolution without being excessive in number. The size of the lipoproteins in a given fraction can then be determined as described below. Generally, the $S_f > 20$ lipoproteins elute in or just after the void volume; those of $S_f = 0 - 20$ elute between 250 to 350 ml; the HDL elute between 350 to 450 ml; and the albumin elution peak is usually near 425 ml.

D. CHARACTERIZATION OF LIPOPROTEIN FRACTIONS

1. Size Determination

A means to confirm the identity of lipoprotein fractions separated by density gradient centrifugation or column chromatography is the determination of the size or diameter of the particles. Laser light scattering, or dynamic light scattering (DLS), is a simple method for determining particle diameter but requires special instrumentation. The Stokes diameter of a particle in solution is calculated by analyzing the autocorrelation function of scattered light fluctuation in a DLS experiment. Aliquots of either whole lymph or lipoprotein fractions are diluted to greater than 1:500 in water and the size distribution of particles within that population measured. Figure 11 shows the distributions for the $S_f > 400$ and $S_f = 20 - 400$ fractions from rat lymph 1 hr after

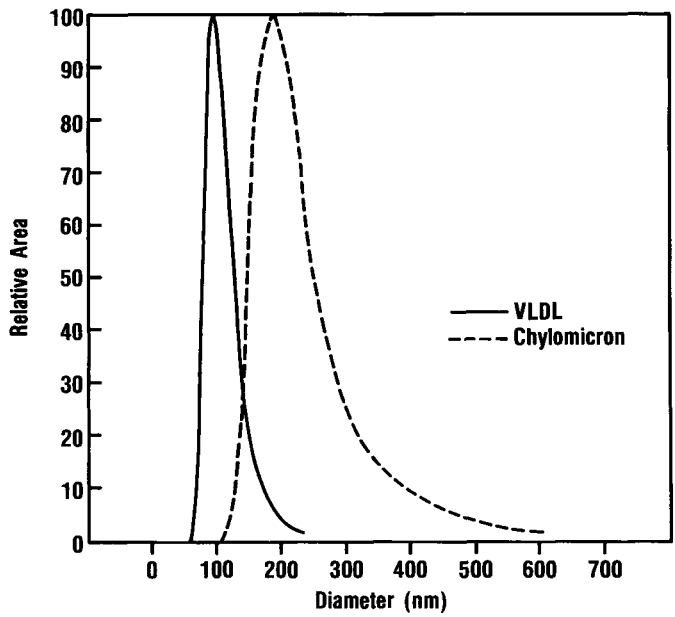


FIGURE 11. Size distribution of rat intestinal lymph fractions as measured by laser light scattering. Lymph was collected during jejunal perfusion with triolein and fractioned by discontinuous density gradient centrifugation into the $S_f > 400$ or chylomicron fraction and the $S_f = 20 - 400$ or very low density lipoprotein fraction. Each fraction was diluted in water and particle size determined using a Nicomp Instruments laser particle sizer.

intraduodenal administration of triolein. The chylomicrons ranged from 120 to 620 nm in diameter with a mean of 170 ± 50 nm and the VLDL ranged from 50 to 250 nm with a mean diameter of 90 ± 20 nm. The significance of these two populations in intestinal lymph is still unresolved. Early work reported that lymph from fat-fed rats contains a continuum of TG-rich particles ranging from 30 to 430 nm in diameter.[58] Havel[65] suggested that lymph VLDL are equivalent to small chylomicrons and that separation of the fractions by sedimentation is artificial; however, recent evidence suggests that each fraction is functionally different and synthesized separately.[66] Lymph HDL particles have a mean diameter of approximately 8 nm.[67]

Another frequently employed method for determining particle size is negative staining followed by electron microscopy. For a general review of this methodology, the reader is referred to the work of Hashmeyer and Myers.[68] In this method, a drop of a diluted lipoprotein fraction is placed on a grid that has been previously coated with 0.25% (w/v) Formvar and its edge touched to filter paper to allow the particles to adhere. Before drying, the grid is inverted onto a drop of stain for a few minutes and is air dried without rinsing. Several different stains are available and we use 2% (w/v) aqueous phosphotungstic acid for 1 min. The image obtained is a negative one where the environment surrounding the particle is electron dense.

Caution must be exercised using this method to determine particle sizes due to potential concerns with standardization and staining. The linear dimensions of particles, measured directly from the photographic negative, can be determined if the electron microscope magnification factor is accurately known. The magnification factor can be determined by photographing an external calibration specimen such as shadowcast silicon monoxide replicas of diffraction line gratings. However, this approach requires identically controlled instrument settings for both the specimen and standard. It is far easier to add an internal standard of known dimension, such as beef-liver catalase crystals[68] or polystyrene latex spheres, to the sample being measured. In addition, air drying of hydrated specimens could lead to significant shape and size artefacts. The same concern arises from penetration of the stain into the specimen which can result in an inaccurate edge-to-edge measurement. Despite these possible drawbacks, we find that this method gives particle diameters for lipoprotein fractions that are equivalent to those measured by light scattering.

2. Compositional Assays

Once the delivery of a compound to the lymph has been demonstrated and the form of association with the lipoprotein fractions has been determined, further work should be done to determine if the delivered compound is present in an unchanged form. It is also instructive to characterize the composition of the lymph and the various lipoprotein fractions with regard to the experimental conditions. It is common practice to use one of the lipoprotein components as a means to normalize the concentration of the compound delivered lymphatically, and in the following sections, we provide some of the basic assays and procedures to measure these components.

a. Phospholipid, Triglyceride, Cholesterol, Total Protein, and Drug

Although the composition (or ratio) of phospholipid, triglyceride, cholesterol, and total protein are different between lipoprotein fractions, it is not possible to utilize composition as a means of identifying the fractions as the fractional composition varies with diet.[69] A typical chylomicron composition includes the following: 86 to 92% triglyceride, 1 to 8% phospholipid, 1 to 2% protein, 0.8 to 1.6% free cholesterol, and 0.8 to 1.4% cholesteryl ester by weight.[70] Diet also has a profound effect on the size of chylomicrons with high fat intake resulting in the formation of larger lipoprotein particles.[69]

We recommend one or more of the following assays, depending upon the nature of the compound being delivered, as a means to normalize the amount of compound present. For instance, intestinal tissue, lymph, or lipoprotein fractions can be extracted following intestinal perfusion and the amount of drug measured as a function of the amount of a component per mole percent, wet gram weight, or volume of sample. A known weight or volume of sample is usually extracted with chloroform-methanol,[71,72] with or without prior acidi-

fication depending upon the ionization properties of the drug. After drying under nitrogen or argon gas, the amount of drug is determined by either total radioactivity, fluorescence, or by HPLC coupled with a suitable detection method.

Phospholipids can be extracted by using acidified chloroform-methanol[71] followed by colorimetric determination of the amount of phosphorous present using the Fiske-SubbaRow reagent.[73] The weight of the total lipid extract is determined after evaporating the chloroform, and the phospholipid content is assumed to be 4% by weight of the total lipid extract. Triglycerides are measured by a colorimetric assay.[74] Cholesterol and cholesteryl esters are quantified by the method of Babson et al.[75] which removes interfering bilirubin.

Total protein content can be measured with any of the standard colorimetric assays, using albumin as a standard, such as the Lowry,[76] Bradford,[77] and Pierce BCA methods.[78] The addition of EDTA to lymph samples can affect the Lowry method, and to a lesser extent the Bradford method. The presence of thimerosal (0.01% w/v) in lymph samples will increase the background of the BCA assay. We prefer the modified Lowry methods because they are suited for samples with a high lipid content.[79,80]

b. Preparative and Analytical Thin-Layer Chromatography

We routinely use thin-layer chromatography (TLC) to identify or recover compound (or metabolites) from lymph and isolated lipoprotein fractions. As an example, we have found that a fluorescent analog of phosphatidic acid, C12-NBD-PA, is converted to C12-NBD-TG within the rat intestinal enterocyte and this metabolite is packaged into lipoprotein particles. Whole lymph or lipoprotein fractions separated by centrifugation are acidified and extracted with chloroform-methanol (9:1) and the organic phase evaporated. The resulting "oil" is diluted in chloroform, spotted onto silica gel GF (250 μ) TLC plates and developed with a solvent system that is appropriate for the objective, such as recovery for further structural analyses (e.g., mass spectrometry) or analytical TLC with standards.

C12-NBD-TG is crudely separated using ethyl acetate:hexane: acetic acid (20:80:1). Ionic species such as the phospholipids are better resolved using a more polar solvent system such as chloroform:methanol:ammonium hydroxide (65:35:5). It may be neccessary, especially for preparative TLC, to perform successive separations with different solvent systems to separate the compound of choice from impurities. For preparative TLC, the C12-NBD-TG is located on the silica gel plate by fluorescence, scraped with a razor blade into a sintered glass funnel and the compound eluted with acetone. The eluting solvent is evaporated and the compound dissolved in chloroform for further purification by TLC in butyl chloride:acetonitrile (85:15) or for quantitation. The utility of this method will certainly depend upon the properties of the compound under study and other approaches, such as HPLC, may be more useful.

III. MORPHOLOGICAL METHODS TO ASSESS INTESTINAL TRANSPORT PATHWAYS

A. INTRODUCTION

By combining perfusion studies with biochemical and morphological approaches it may be possible to identify the route by which compounds traverse the intestinal epithelium. Localization of the compound of interest is also essential for a general understanding of the function, mechanism of action, site of action, and clearance. Such information becomes important when trying to enhance the absorption of compounds either by structural modifications or by addition of penetration enhancers or adjuvants. In the case of lymphatic targeting, a compound may enter the lymph alone or in association with lipoprotein particles.

The association of orally administered compounds with lipoprotein particles may occur in three ways. A compound can diffuse through the epithelial cell membrane and be packaged into the lipoprotein particles during their synthesis via the lipid absorption pathway. Alternatively, a compound can diffuse through or between the epithelial cells and either partition into the forming and/or mature lipoprotein particles by virtue of the compound's intrinsic partition coefficient, or bind to components that compose the surface of the lipoprotein particle. The difference in efficiency of these routes is unknown; however, due to physical constraints, substitution of an endogenous substrate with an orally administered compound would be expected to have a higher capacity than if the compound simply partitioned into a mature lipoprotein particle as discussed by Urien.[81]

For most methods, determining the distribution of a compound requires complete and irreversible immobilization of the agent within the tissue. An exception is confocal laser scanning microscopy which, when coupled with epifluorescence, could be used to localize compounds within living tissue and cells (see Section III D). It is the requirement for immobilization that limits the precise localization of small hydrophilic or lipophilic compounds such as conventional drug molecules. These substances are usually not reactive with the commonly used chemical fixatives and, therefore, are readily extracted during tissue processing for standard histological methods and undergo diffusion within the tissue. The localization of diffusible substances has been an area of frustration and most of the pioneering advances have been accomplished by using autoradiographic approaches, especially for the localization of lipids and steroid hormones.[82–84] In the following sections, we review some methods for localizing lipophilic compounds with relevance to lymphatic delivery and discuss conditions under which these approaches should be the most helpful.

B. TISSUE PREPARATION

The usual cascade of treatments to prepare biological samples for histology (i.e., fixation, dehydration, embedding, and sectioning) is often detrimen-

tal to the localization of diffusible substances. Chemical fixation or precipitation, which is critical for preventing subsequent extraction or redistribution, is only useful when the compound being studied has the appropriate reactive groups. Many of the dehydrating agents and the hydrophobic embedding resins are excellent lipid solvents. Consequently, particular attention must be paid to the details of sample preparation since the validity of the results are dependent upon that step where loss or diffusion of the compound most likely occurs. In the following sections, we highlight some of the strategies for chemical fixation or the alternative of cryofixation and the use of embedding media.

1. Chemical Fixation

It is imperative that the physicochemical properties of the compound under study and the underlying fixation chemistry be considered when designing a strategy for tissue or cellular localization. There are relatively few specific chemical fixatives available, and ideally an agent is selected that can form a bridge between reactive groups on the compound of interest and surrounding proteins that are co-fixed. Fixation is not only important for retention of the compound under study, but also for preservation of the tissue and cellular structures. The best performing and most commonly used fixation, especially for electron microscopy, is sequential immersion in buffered solutions of glutaraldehyde (glutaric acid dialdehyde) and osmium tetroxide. While glutaraldehyde and formaldehyde work well in the fixation of proteins, they are of limited use for fixation of lipids, and their use alone will not prevent subsequent extraction. For example, after fixation with glutaraldehyde, most of [^{14}C]triolein and [^{3}H]palmitate were lost to the ethanol solvent during dehydration. However, inclusion of osmium tetroxide during fixation improved their retention.[83,85,86] Retention may be indirectly improved by entrapment within the surrounding fixed environment, although this may be inconsequential for small molecules that are weakly hydrophobic.

Calcium-formol is widely used as a general fixative for lipid histochemistry. The solution is buffered with 2% (w/v) calcium acetate to prevent acidification of the formol solution as a result of oxidation of formaldehyde to formic acid during storage. This fixative appears to preserve phospholipids[87] but not neutral lipids, and the fixation may occur through ionic bridging or crosslinking of the negatively-charged head group with nearby cationic moieties. Formaldehyde used alone is not an ideal fixative since it is a monoaldehyde which is incapable of crosslinking and is reversible in aqueous solvent.[88] It is interesting that calcium ions may help retain fatty acids by altering their solubility in organic solvents through the formation of insoluble salts,[89] and this raises the possibility of using additives to precipitate compounds of interest without requiring a covalently-linked complex. Digitonin has been used in this manner to form stable and insoluble precipitates with the 3β-OH group of steroids and cholesterol.[90] When combined with simultaneous fixation with glutaraldehyde/osmium tetroxide and subsequent dehydration, localization of cholesterol, but not cholesteryl ester, is possible.[91]

Aldehydes react with primary amine groups (e.g., arginine, glutamine, histidine, lysine, tryptophan) and sulfhydryl groups (e.g., cysteine) to form a stable Schiff base.[92] Methylene bridges may form more slowly and reversibly and this may include amide groups.[88] Each aldehyde differs in the rate and extent of its reaction with these groups and this will be dependent upon the diffusion coefficient, duration of fixation, solution pH, temperature, tonicity, and the spatial orientation of the reactive group. The impact of each of these factors is dependent upon the characteristics of the tissue. The addition of nonelectrolytes, such as sucrose and dextran, to the fixative have been shown to increase protein extraction,[93,94] but their effects on lipophilic substances are unknown. In general, increased temperature results in faster penetration and faster reaction of the fixing agent, but it also increases the extent of extraction. Therefore, it is generally best to fix these samples at 4°C. If a radiolabeled compound is available, it is advisable to monitor the loss of radioactivity with each step of sample preparation and to demonstrate that the retained radioactivity is parent compound. We find that for highly lipophilic [^{3}H]compounds ($\log P > 6$) of low aqueous solubility (< 50 ng/ml) less than 10% is lost during fixation with glutaraldehyde at room temperature for 30 min (Raub, unpublished results).

The other commonly used fixative, especially for lipids, is osmium tetroxide; however, despite its accepted utility for the retention of lipids the fixation is not absolute. The chemistry of osmium fixation is complex and less well understood than the other fixatives. Osmium tetroxide oxidizes aliphatic double bonds with 1:1 stoichiometry to yield the corresponding vicinal cis-diol which then reacts with monoester osmic acid to form a crosslinked diester.[88,95] It also reacts with tertiary amines (e.g., pyridine, quinoline)[96] and some aromatic hydrocarbons (e.g. anthracene) by attacking at sites of highest electron density[97] and ethylene bonds.[88] Reaction of osmium tetroxide with imines and nitriles is not generally observed.[96]

As with aldehydes, osmium fixation is dependent upon the factors discussed above, and although it is a relatively poorly penetrating agent with moderate reactivity it is an excellent fixative when used in combination with glutaraldehyde. In regions of high lipid deposition such as lipid droplets and lipoprotein particles, osmium initially reacts with the outer surface components forming a shell which limits its subsequent diffusion.[88] Consequently, the inner region of these structures is often poorly fixed.

Spectroscopic methods demonstrate that glutaraldehyde at concentrations greater than 2% (w/v) has a profound effect on the mobility of proteins in biological membranes but not on lipid movement.[98] Osmium tetroxide (1 to 2% w/v) has a profound effect on the mobility of unsaturated lipids, no effect on saturated phospholipids with the possible exception of phosphatidylserine,[88] and most likely reduces the lateral diffusion of most constituents within biological membranes.[98] Despite these results, simultaneous fixation with glutaraldehyde and osmium tetroxide still results in a loss of approximately 70%

[^{3}H]oleic acid during dehydration, although this is a significant improvement over the quantitative loss following sequential fixation with glutaraldehyde and osmium tetroxide.[99] It is important to mention that the fate of endogenous lipids and absorbed lipids during processing may differ.[100]

Osmium tetroxide as a primary fixative has also been used to retain compounds via precipitation in an analogous manner to the use of divalent cations and digitonin to retain fatty acids and cholesterol, respectively. It is proposed that osmium-compound complexes can form, which by virtue of their insolubility and electron density, allow for the direct localization of compounds which had diffused into the tissue.[101] However, these results must be viewed cautiously since it was not confirmed that the precipitate contained the compound under study.

The best approach is to avoid immersion fixation unless a known chemical reaction, preferably one of crosslinking, is predicted between the fixing agent and the compound being localized. Results will be obtained whether or not the fixation chemistry is appropriately considered; however, it is very difficult to determine whether they are valid in light of the potential for diffusion. To further complicate matters, chemical fixation results in tissue and cellular shrinkage,[88] and this may also influence distribution of the compound. Many of these problems can be overcome by the application of cryo methods discussed in the following section.

2. Cryofixation

The method that has proven to be the best approach for localization of diffusible substances by microscopic autoradiography (see Section III C) uses frozen sections. In this manner the exposure of samples to dehydrating agents and infiltration for embedding, during which extraction and diffusion are significant, is avoided. Tissue samples are snap frozen either unfixed or following fixation by immersion into isopentane or propane chilled by liquid nitrogen. When using a fixative, we have the best results with Brenner's fixative containing 2.2 m*M* 2,4,6-collidine.[102] We mount 2 to 4 mm thick crossections of small intestine onto cork disks using a drop of Tissue Tek O.C.T. (Miles Laboratories, Napperville, IL) or M-1 Embedding Matrix (Lipshaw, Detroit, MI) and immerse it for 30 sec into isopentane at –150°C (–180°C for propane). The samples are transferred to boxes and stored at –70°C. The samples are equilibrated for 0.5 to 1 hr in the cryostat at the desired sectioning temperature and 5 to 10 μm sections are obtained. For the best results, each tissue has an empirically determined optimal temperature which is usually between –10° and –20°C. Generally, soft tissues are cut at relatively higher temperatures than fibrous tissues, as are fixed vs. unfixed tissue. We cut unfixed intestinal tissue at –18°C.

It is now possible to routinely obtain thin (0.5 to 1.0 μm) cryosections for high resolution light microscopic autoradiography (LM-ARG) or fluorescence microscopy and ultrathin cryosections of < 100 nm for electron microscopic

ARG.[103–105] While this technology is generally used in immunocytochemistry, its application in EM-ARG has been suggested as a potentially major breakthrough for the localization of diffusible substances.[106–108] In these procedures, the sample is usually fixed and infiltrated with a cryoprotectant (e.g., sucrose) prior to freezing, although metal block freezing of unfixed material can also be used. For greater detail, the reader is referred to the original literature.[109–111]

Another cryo approach employs freeze-substitution which has been applied to the localization of diffusible ions such as potasium. It is likely that retention of diffusible ions is facilitated by ionic interaction with cellular macromolecules.[112,113] In this method, frozen samples are dehydrated at low temperature in an organic solvent such as ethanol, methanol, or acetone which is then subsequently replaced by a resin which is polymerized at low temperature.[114] While this approach has the disadvantages of dehydration and embedding for extraction and diffusion of compounds, it offers the advantage of reduced temperature, which will lessen these problems, improve morphology and applicability to electron microscopy.

Depending upon the method of choice, the sections are handled in a variety of different ways as described in Section III C. Selection of a method depends upon the diffusibility of the compound under study. For instance, highly diffusible substances can not tolerate a rise in temperature or prolonged exposure to aqueous solutions.

3. Embedding Media

Probably the most deleterious steps in tissue processing to the successful localization of diffusible substances are dehydration and embedding, since most of these agents are excellent lipid solvents. There are some changes that can be made to minimize the potential for extraction. As discussed in Section III B.1, the degree of loss is dependent upon the duration of incubation, temperature, pH, and the solvation characteristics of the agent. It has been suggested that dehydration in acid-alcohol will preserve lipids,[115] although this is mostly for phospholipids.[88] It is recommended that all steps be done with agitation to reduce the duration of the procedure and be performed at 0°C to minimize extraction. If organic solvents are used, selection is based on the relative solvating properties, and consequently methanol is a better choice than ethanol as it is an inferior solvent. Ethylene glycol or dimethylformamide also can be used. Propylene oxide, which is commonly used in electron microscopy, is a good solvent for even fixed lipids due to its reactive properties and should be avoided. Ideally, if the compound is lipophilic and of low aqueous solubility, it is best to avoid organic solvents and use water-miscible agents to reach the same objective.

The epoxy resins that are typically used for electron microscopy are hydrophobic and themselves are good solvents of lipophilic substances. This is true even for the monomers of some of the water-miscible embedding polymers,[88] which are preferred for the purposes of low extraction. This

approach can be successful when used in conjunction with low temperature infiltration and polymerization, and in fact, the embedding medium itself can be used in lieu of a dehydrating agent. Extraction of [^{3}H]oleic acid was improved 40% by dehydrating in dilutions of glycol methacrylate;[99] however, even this water-miscible embedding medium is not suitable for the retention of neutral glycerides.[88] The use of a water-soluble polymer of glutaraldehyde and carbohydrazide (GACH)[116] resulted in 90% retention of [^{3}H]palmitic acid following dehydration and embedding.

Due to the problems associated with embedment for the immunocytochemical localization of antigens, many new embedding media have been introduced (see products carried by Polysciences, Inc., Warrington, PA). There are a variety of hydrophilic, polar, and apolar acrylic-based polymers (e.g., LR Gold, LR White, Lowicryl) that are poor lipid solvents. Many of these media can be polymerized at low temperature which may further minimize problems with diffusion.[114,117] More studies using these media need to be done to assess their usefulness in the localization of diffusible compounds by light and electron microscopy.

C. AUTORADIOGRAPHY

The most common approach for localization of diffusible substances within tissue and cells is microscopic autoradiography. A considerable amount of work has been done in perfecting this technique over the past 40 years and several excellent reviews have been written.[82–84,101] It is not the intention of this section to review all of the details of this method but rather to highlight the relevant criteria.

Autoradiography is the production of an image in photographic emulsion by radiation emitted from a source in tissue that is in close contact with the emulsion. In addition to the problems discussed so far, results are dependent upon the properties of the isotope (e.g., particle energy, specific activity), section thickness, and emulsion properties. In general, sections of less than 1 μm in thickness give good results since most of the low energy isotopes ([^{3}H], [^{14}C]) that are commonly used to label compounds travel relatively short distances. However, temperatures of less than –40°C are required to obtain sections of this thickness. Therefore, in thicker sections it is only those radiolabeled molecules near the surface of the section which produce an image. Although these low energy isotopes give good resolution, there is the potential for quenching to occur within the tissue.

There are a variety of ways in which sections are handled to produce the autoradiogram and the choice of a particular approach is dependent upon the compound under study. If the substance is immobilized, then sections are mounted onto gelatin-coated slides (0.2% w/v aqueous solution of gelatin containing 0.02% v/v chromium potassium sulfate) which are then dipped in liquid emulsion.[118,119] We have used this approach to localize a renin inhibitor hexapeptide (RIP, BOC-Pro-Phe-N-Methyl(His)-Leu-ψ-($CHOHCH_2$)Val-Ile-

AMP[120]) following perfusion of the rat small intestine (Figure 12). Since appearance of [^{3}H]RIP within the mesenteric vein was tenfold less than the amount which disappeared from the perfusate, we used LM-ARG to examine its distribution in the lumen and surrounding intestinal tissue.[121] This approach was successful because of the presence of a histidine residue within the RIP molecule which reacted with aldehyde[88] and most likely served to immobilize the RIP following glutaraldehyde fixation.

In another study, the distribution of a cationic ($pK_a = 6.5$) highly lipophilic compound ($\log P > 8$) without "fixable" groups within a cultured epithelial cell monolayer at pH 7.4 was determined by LM-ARG (Figure 13). The cryosections were thawed, air dried and then dipped in liquid emulsion. Although distribution of the compound within the plasma membrane is dominant, there is still evidence for diffusion despite poor intrinsic aqueous solubility of the compound.[122]

If the substance under study is diffusible then special precautions must be taken. The two basic methods involving cryosections, which are used to avoid specimen contact with liquids (solvents and liquid emulsion), are dry mounting and thaw mounting,[84] and both methods require the sectioning to be performed in a darkroom and the sections to be applied to the emulsion-coated slides. Thaw-mount ARG is done by picking up the cryosections onto emulsion-coated slides that are slightly warmer than section temperature. The melting of the section facilitates adherence but this is a condition under which diffusion can occur. Dry-mount ARG is accomplished by drying the cryosections under vacuum ($< 5 \times 10^{-5}$ mm Hg) such as cryosorption or freeze-drying,[84] for 20 to 24 hr and placing the dried section on an emulsion-coated slide. This latter method has been shown to give superior results compared to thaw-mount air-dried frozen sections dipped in liquid emulsion, and freeze-drying followed by embedment for localization of [^{3}H]estradiol and [^{3}H]mesobilirinogen.[123] The thaw-mount technique is most common[124] and applications of both approaches, including those with slight variations, are reviewed by Stumpf and Roth,[125] and Stein and Stein.[83]

D. FLUORESCENCE MICROSCOPY

Another methodology that can be used to localize drugs within tissues is that of epifluorescence microscopy. The major limitation with this technique is that the compound of interest must be fluorescent, which is accomplished due to either native fluorescence or by derivatization with a suitable fluorophore. The paucity of examples describing the localization of compounds with endogenous fluorescent properties is most likely because of the rarity of these compounds and the limitations imposed by the method. However, with the availability of laser light sources and improved variable range imaging, it is anticipated that this approach will have wider applicability. When combined with the relatively new technique of scanning confocal microscopy[126] where a

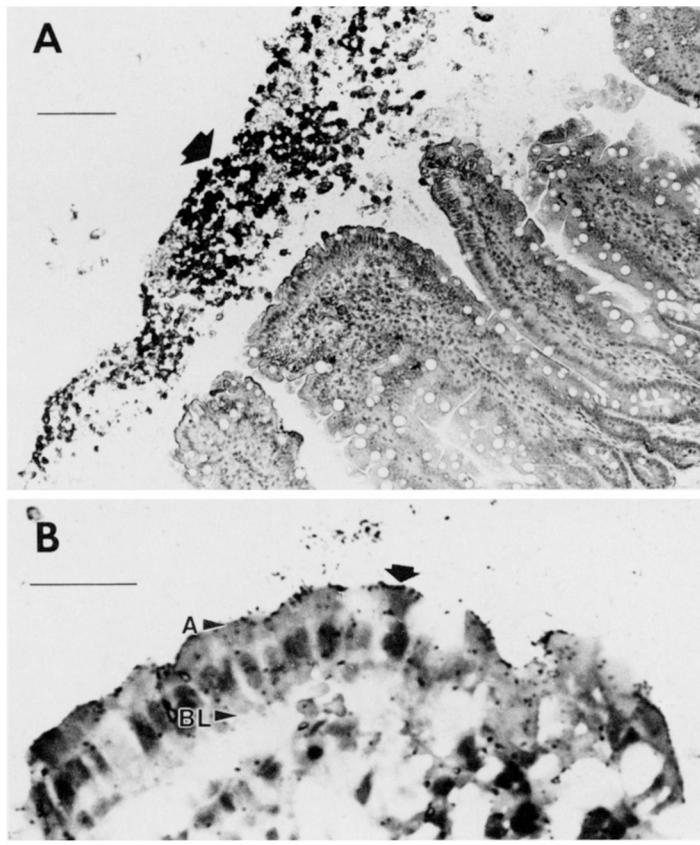
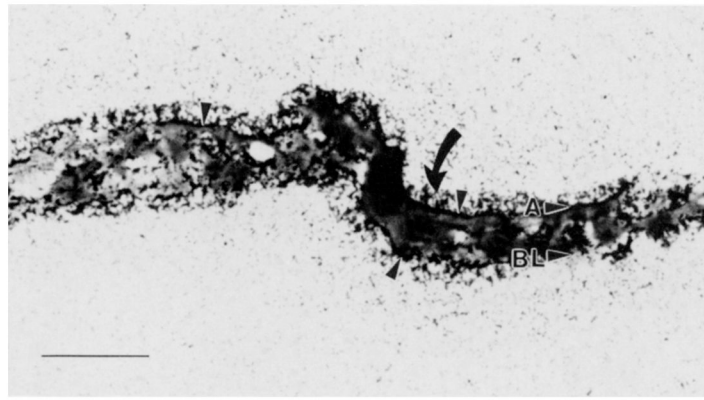


FIGURE 12. Light microscopic autoradiography of rat jejunum following perfusion for 1 hr with a [^{3}H]renin inhibitory peptide (structure shown) in phosphate buffer at pH 6. (A) The majority of the labeled peptide is associated with the mucus layer (arrow) overlying the villi. Bar equals 100 μm. (B) At higher magnification, a small fraction of the labeled peptide (arrow) is adsorbed to the apical (A) or lumenal surface of the intestinal epithelium. Basolateral or abluminal surface, BL. Bar equals 25 μm. The perfused intestine was excised, fixed by immersion in glutaraldehyde, and then frozen. Cryosections were air dried, dipped in liquid (1:1) Ilford K.5D emulsion and exposed for 2 to 3 weeks.



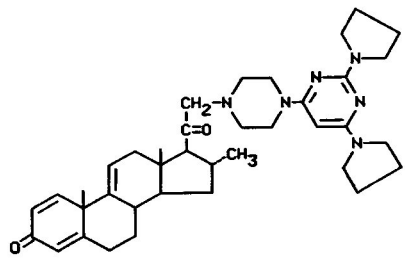


FIGURE 13. Localization of a highly lipophilic [^{3}H]labeled compound (structure shown) within a cultured epithelial cell monolayer. The cell monolayer, grown on a polycarbonate filter which has been dislodged, was exposed at the apical surface (A) to radiolabeled compound for 5 min at pH 7.4. The cell monolayer was rinsed, rapidly frozen and light microscopic autoradiography performed as described in Figure 12. While much of the labeled compound appears to be associated with the plasma membrane (small arrows), a significant portion has diffused from the cells during sample handling. Bar equals 25 μm.

spatial specimen image is constructed by raster scanning of a focused laser source at 200 nm resolution,[127] this dual approach could provide a great deal of information about compound distribution in living samples. For example, Rojanasakul and co-workers[128] recently used this approach to examine the diffusional pathway of fluorescently derivatized proteins across unfixed corneal epithelium.

Derivatization must be considered with caution since addition of a fluorophore will increase the molecular weight and may significantly alter the physicochemical properties and subsequent distribution of the parent compound. If this approach is used, it is critical to demonstrate that the fluorescent analog behaves similarly to the parent compound with regard to its biological and physicochemical properties. Under these circumstances, a fluorophore can be selected with characteristics that allow its visualization by conventional fluorescence microscopy. Such an approach has been utilized to elucidate the trafficking and metabolism of fluorescent lipid analogs within cells.[129]

Most of these studies employ the NBD fluorophore (N-(7-nitrobenz-2-oxa-1,3-diazole) that is linked to the glycerol backbone at the C2 position through an acyl spacer group. NBD-cholesterol, which appears to mimic native cholesterol when assessed by a number of physical and biological criteria, has been used to label lipoprotein particles *in vitro* for examining their subsequent interaction with cultured cells.[130,131] We have combined both approaches to examine the intestinal absorption and delivery of NBD-lipid analogs into lymph via their incorporation into lipoprotein particles.[62,63] As an example, NBD-PA, where PA represents a model lymphotropic carrier molecule to which is conjugated a model "drug like" molecule (e.g., NBD), is treated by the enterocyte as a dietary lipid and is presumably packaged into lipoprotein particles during their synthesis.

The NBD-PA is localized in the small intestine mucosa by treating it as a diffusible substance and using a cryofixation approach. Tissue is snap frozen and 5 μm cryosections mounted onto glass slides at −18°C. The sections and slides are not stored, but are removed from −20°C, coverslipped at 4°C with 75% (v/v) glycerol in phosphate buffered saline containing 1 mg/ml p-phenylenediamine[133] to retard quenching, and viewed immediately with epifluorescence. Since NBD has the same spectral properties as fluorescein (EX_{max} = 465 nm and EM_{max} = 545 nm) the same filters can be used. The image must be immediately photographed to avoid diffusion artifacts as the sections are subject to thawing in the aqueous milieu. One hour after infusion of NBD-PA in corn oil into the duodenum of rats, fluorescence was most concentrated in inclusions within the epithelial cells at the tips of the villi (Figure 14) which is consistent with the lipid absorption processes. Fluorescent particles are also present within the lamina propria, presumably within the central lacteals (Figure 14C), and within lymph collected from the mesenteric lymph duct.

Dual labeling procedures can be employed to further identify the fluorescent structures. An area is photographed which is marked using micrometer coordinates on the microscope stage and the section is retreated with a lipophilic stain. For example, NBD-fluorescence was correlated with Nile red fluorescence by recoverslipping in glycerol containing 2.5 μg/ml Nile red which is a phenoxazone dye that only fluoresces when in association with neutral lipid deposits including unesterified cholesterol (Figure 15).[134,135] The same structures can also be identified as osmiophilic by placing the slide with section with several drops of 1 to 2% osmium tetroxide in a sealed Petri dish for 0.5 hr.

Some success has been obtained in localizing the subcellular distribution of NBD-lipids and other fluorescent molecules[136] within cultured cells by using electron microscopy.[137] This has been accomplished by photoconversion of the fluorescence to a diaminobenzidine product.[138] In this system, fixed cells are irradiated in the presence of 1.5 mg/ml diaminobenzidine tetrahydrochloride in 0.1 *M* Tris buffer (pH 7.6) for 30 min using a low magnification objective and either a 100 W mercury lamp[136] or an argon laser.[137] After osmication, the

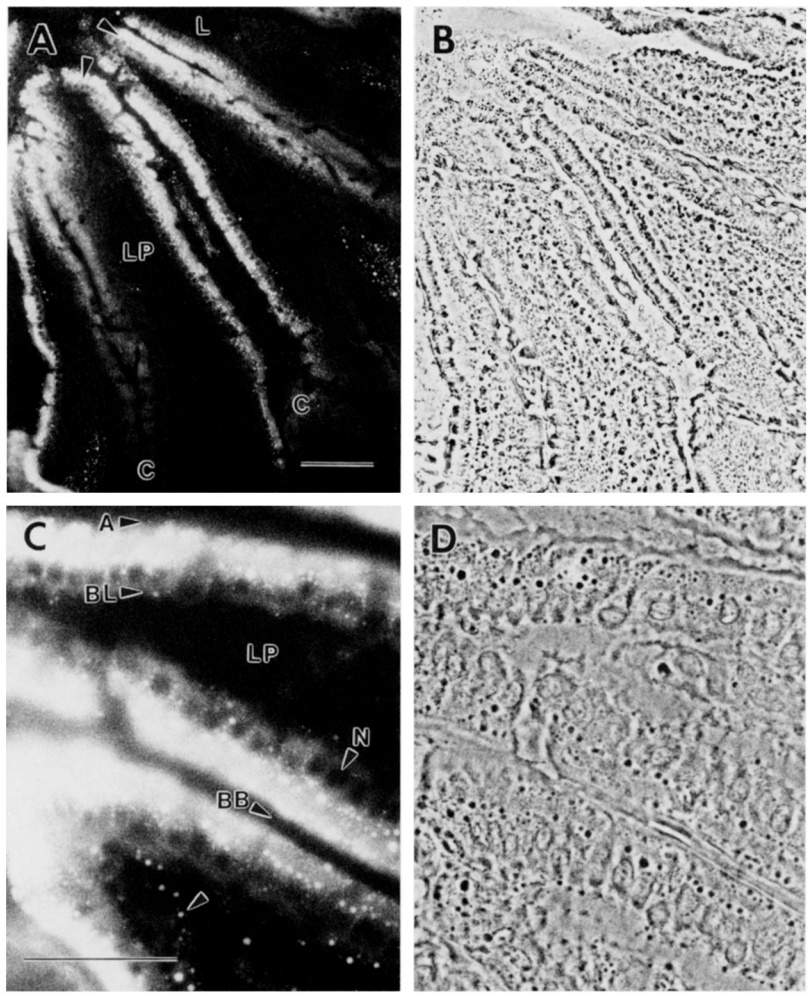


FIGURE 14. Distribution of a fluorescent analc ; of phosphatidic acid (see Figure 5 for structure) within rat jejunum following closed perfusion .or 1 hr with triolein. (A) Fluorescence is most intense within the epithelium at the tips of the villi (arrows). Lumen, L; lamina propria, LP; crypts, C. (B) Phase contrast image of (A). Bar equals 100 μm. (C) Higher magnification of the jenunal epithelium showing that the absorbed fluorescent compound is localized within droplets that predominate within the apical (A) cytoplasm of the enterocyte. Fluorescently labeled particles are also present within the lamina propria (LP) at the basolateral (BL) surface of the epithelium and presumably within the lacteal (arrow). Brush border, BB; nucleus, N. (D) Phase contrast image of (C). Bar equals 50 μm. Following brief perfusion with glutaraldehyde, intestine was excised and rapidly frozen. Cryosections (8 μm thick) were mounted and stored at –20°C and photographed as they warmed to room temperature to minimize diffusion.

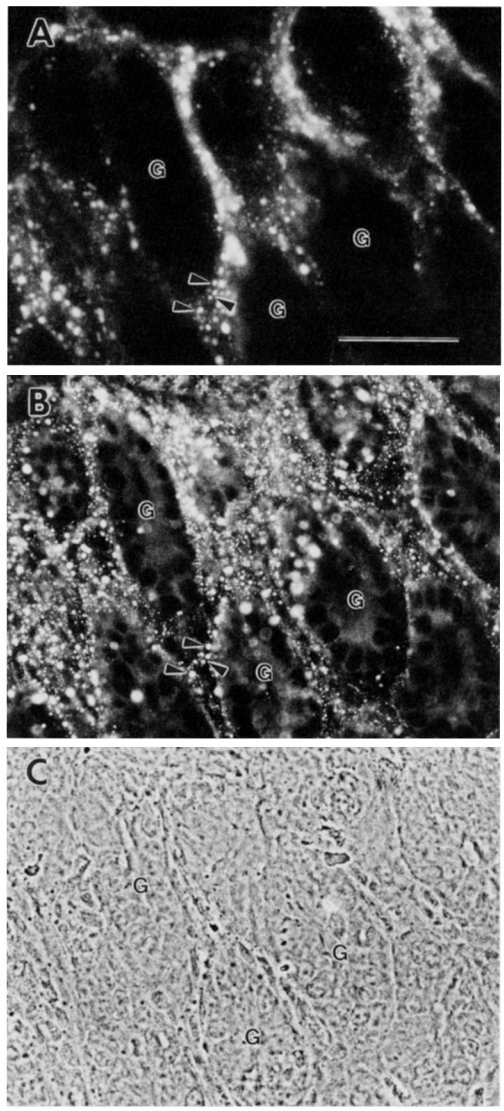


FIGURE 15. Co-localization of a fluorescent analog of phosphatidic acid with neutral lipids and unesterified cholesterol using the lipophilic dye Nile red within rat intestinal tissue. (A) Fluorescently labeled particles (emission of 520 to 560 nm) are found in the perifollicular plexus of the lymphatics, or in the interstitial space between glands (G) within the tunica submucosa, following closed perfusion of rat jejunum with C12-NBD-PA in triolein. (B) After photography, the tissue is recoverslipped with mounting medium containing Nile red and the fluorescence of this dye (emission > 590 nm) recorded for the same field of view. The arrows denote particles of coincidence. Not all particles are mutually fluorescent. (C) Phase contrast image of (A) and (B). Bar equals 50 μm.

samples are processed for transmission electron microscopy. This procedure results in an electron-dense deposit at the site of the fluorescent molecule. The success of the photooxidation procedure is dependent upon the intensity of the fluorescence and the light source. This approach is also subject to the same concerns in immunoperoxidase cytochemistry regarding diffusion of the DAB product.[139] To our knowledge, this method has not been applied to tissue and may prove useful for the ultrastructural localization of NBD-lipids and other fluorescent compounds within intestinal mucosa.

IV. SUMMARY

The intestinal lymphatic transport of drugs and the potential for subsequent targeting are compelling reasons for conducting studies in this area. This chapter has outlined some of the varied and different methodolgies which the authors have found useful in evaluating the potential of intestinal lymphatic drug transport. Due to the complex nature of lipoprotein synthesis and the drug transport process, the investigation of intestinal lymphatic transport will be enhanced by an integrated approach which considers transport on a cellular level in conjunction with the overall context of the experimental animal model. Through the application of the techniques described in this chapter (or similar methodologies), it should be possible to more fully and comprehensively evaluate the potential and role of intestinal lymphatic drug transport.

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Chapter 4

LIPID VEHICLE AND FORMULATION EFFECTS ON INTESTINAL LYMPHATIC DRUG TRANSPORT

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I. GENERAL CHARACTERISTICS OF INTESTINAL LYMPHATIC TRANSPORT

The intestinal lymphatic system comprises a network of structures throughout the small and large intestine which have a major physiological role in the absorption of a variety of nutrients, fluids, and lipids.[1] In particular instances, the intestinal lymphatics may significantly augment the systemic bioavailability of orally administered drugs.[2–4] The absorptive capacity and functional characteristics of the lymphatics in the small and large intestine are different, and this regional specificity translates to distinct absorption and transport profiles of drugs and nutrients. The features of the lymphatics present in the large intestine and their role in the absorption and transport of polar drugs is addressed in Chapter 7. This chapter is restricted to a description of the small intestinal lymphatics and its involvement in the absorption and transport of orally administered lipophilic compounds to the systemic circulation.

Centrally located lacteals which are present within the intestinal villi join a plexus of lymphatic capillaries in the mucosa and submucosa and drain, via the mesenteric lymph vessel, into the cisterna chyli. This structure also collects hepatic and regional lumbar lymph. Lymph from the cisterna chyli is drained by the thoracic lymph duct which empties directly into the general circulation at the junction of the left internal jugular and left subclavian veins. This unique physiology enables orally administered drugs which are transported by the intestinal lymphatics to bypass the liver prior to reaching the general circulation and therefore avoid a potential "first pass effect".

A. PHARMACEUTICAL ASPECTS OF INTESTINAL LYMPHATIC DRUG TRANSPORT

From a pharmaceutical standpoint, the promotion of intestinal lymphatic drug transport may enable the oral administration of compounds whose

bioavailability had otherwise been limited by significant first pass metabolism. Section II of this chapter addresses the likely contribution of intestinal lymphatic transport to the enhanced bioavailability of poorly water soluble drugs which is often observed when administered in combination with a lipid vehicle or in the presence of food. Additional pharmaceutical advantages generally ascribed to the enhancement of intestinal lymphatic drug transport are (i) the potential to direct the delivery of chemotherapeutic agents to appropriate segments of the lymphatic system which are otherwise refractory to intravenous chemotherapy,[5–7] and (ii) the controlled rate of delivery of orally administered drugs to the systemic circulation.[3,8]

The intestinal lymphatics are generally considered a minor pathway of drug absorption and subsequent transport except for highly lipophilic molecules such as the natural lipids, lipid soluble vitamins, and certain drugs and xenobiotics. Examples of lipophilic drugs in which intestinal lymphatic transport has been demonstrated include cyclosporin,[9] naftifine,[10] probucol,[11] various vitamin esters and derivatives,[12] some hypolipidemic agents,[13] numerous xenobiotics including DDT and associated analogs,[14] benzopyrene,[15] and polychlorinated biphenyls (PCBs).[16] A common characteristic of lymphatically-transported drugs and xenobiotics is their lipophilicity, generally typified by a large partition coefficient between an organic solvent, such as n-octanol, and an aqueous phase (e.g., logarithm of partition coefficients at least 5 to 6 at physiological pH). A high partition coefficient of a drug is required, but not necessarily sufficient, for ensuring significant intestinal lymphatic drug transport. Additional and necessary physicochemical and biological factors include adequate lipid solubility, absorbability from the intestinal lumen, and metabolic stability within the enterocyte.[4,17] These factors will be discussed in Sections III and IV.

II. DRUG BIOAVAILABILITY AND LIPID VEHICLES

The effects of lipids on the bioavailability of orally administered drugs are highly complex due to the numerous mechanisms by which lipids can alter the biopharmaceutic and pharmacokinetic characteristics of the administered drug.[2,18–21] The effects of orally administered lipid can include (i) a decreased rate of gastric emptying, (ii) an increased dissolution rate of the drug, (iii) an enhancement of the solubility of the drug within the intestinal milieu through solubilization, and (iv) the formation of lipoproteins which provide a means of transport for those drugs/xenobiotics in which lymphatic transport may be a contributor to the bioavailability of the drug. The effects of lipids on gastric emptying and lipoprotein synthesis are direct in nature,[22,23] whereas the role of lipids in drug solubilization and dissolution are a result of lipid digestion (especially in the presence of food) and the stimulation of biliary and pancreatic secretions.

Due to the wide range of effects which lipids can elicit within the gastrointestinal tract, co-administered lipid will influence the absorption process of

TABLE 1
Different Mechanisms by Which Orally Administered Lipids Can Potentially Enhance the Bioavailability of Poorly Water Soluble Drugs via Either the Portal Blood or Intestinal Lymphatics

Effects of lipid	Portal blood	Intestinal lymphatics
Enhanced dissolution	+	+
Solubilization	+	+
Increased residence time	+	+
Lipoprotein formation	–	+

drugs via the portal blood and also the transport of highly lipophilic drugs which are transported via the lymphatic system. Effects of lipid on drug absorption via the portal blood route are a likely manifestation of the changes in gastric residence time and the enhancement of solubility and dissolution rate of lipophilic drugs within the intestinal milieu. The effect of lipids on drug bioavailability is often determined from drug plasma concentration-time data arising from pharmacokinetic studies, and it is therefore difficult to differentiate between the intrinsic effects of the lipid upon absorption via the portal and/or lymphatic routes. Table 1 lists the common mechanisms by which co-administered lipid may enhance the absorption of poorly water soluble drugs via either the portal blood or intestinal lymphatics.

Although the effects of lipids on the bioavailability of drugs are not regularly studied per se, many of the fed-fasted studies which are conducted have a significant lipid component present in the co-administered food. The effect of food on the bioavailability of orally administered drugs is regularly studied in humans on most new drugs and oral dose forms during their development. Although the data from these studies (drug plasma concentration-time profiles in the fed and fasted states) are instructive in terms of the potential effects of food on the bioavailability of the particular drug, they often do not provide useful data regarding the mechanisms involved in the so-called "food effect". Consequently, it is difficult to determine the intrinsic effects of the lipid/food on either the luminal-related processes (solubilization, dissolution and residence time) or the enterocyte-related processes (lipoprotein formation) which may contribute to the enhanced bioavailability of the drug.

A. CHARACTERISTICS OF LYMPHATIC TRANSPORT

If a lipophilic drug/xenobiotic is significantly transported by the intestinal lymphatics in association with lipoproteins, it would therefore be expected that the co-administration of food containing an appropriate lipid would increase the bioavailability of the drug relative to the fasted state, i.e., the lipid would provide the means for the formation of chylomicrons by the enterocyte. However, the converse is not necessarily the case — an increased bioavailability of drug due to co-administration of food does not have to involve lymphatic drug transport, i.e., the lipid-related effects may have led to an increase in absorption

of drug via the portal blood through enhancement of bioavailability. Although the terms lymphatic transport and lymphatic absorption are often used interchangeably in the literature, lymphatic transport is used exclusively in this chapter as it more clearly implies the involvement of a transport system (lipoproteins) rather than an absorption process per se.

Although it is possible to infer lymphatic transport of a drug by the judicious choice of the co-administered lipid vehicle,[11,24] confirmation of intestinal lymphatic transport requires quantitation of the drug content in collected mesenteric lymph. The confirmation of intestinal lymphatic transport requires utilization of appropriate animal models as it is not possible to routinely collect samples of intestinal lymph from humans. Chapter 3 addresses the methodological issues and describes the features of various animal models which can be utilized for the assessment of intestinal lymphatic drug transport. As lymphatic transport can only be reasonably proven in an animal model, attempts at extrapolation and comparison of these transport data to the human situation must be tempered by the realization that only indirect measurements such as drug plasma concentration-time profiles or urinary excretion data will be available from the human. These measurements will reflect the combined drug absorption via the portal blood as well as potential transport via the intestinal lymphatics. Additionally, differences in the gastrointestinal anatomy and physiology between experimental animals and the human require consideration when extrapolating animal data. For example, the rat, which is a common model for assessing lymphatic transport, does not have a gall bladder and consequently the factors controlling the flow of bile between rats and humans are different. Due to differences in size and capacity of the gastrointestinal tract between experimental animal models and man, the efficiency of lipid digestion and emulsification as a function of the administered volume will no doubt be different, which further complicates attempts at extrapolation of animal data. Furthermore, the rat is generally considered to be more metabolically active than humans, which makes the extrapolation of lymphatic transport data more difficult.

As a general guide, a "typical idealized" plasma concentration-time profile of a "typical highly lipophilic drug" which is predominantly transported by the intestinal lymphatics may include (i) a delayed time for attainment of maximal drug plasma concentrations (Tmax) reflecting the slow lipoprotein-based input of drug into the systemic circulation relative to portal blood absorption, and (ii) a "flat" drug plasma concentration profile due to the continued formation of lipoproteins and transport of drug to the systemic circulation (and not an intrinsically slow elimination half-life of the drug). The magnitude of Tmax, and the plasma profile characteristics of the drug, are dependent upon the relative contributions of lymphatic transport and portal blood absorption as well as the potential for metabolism by the enterocyte.[25]

The following brief comments highlight points for consideration when interpreting intestinal lymphatic transport data. Intestinal lymph is transported via the mesenteric lymph duct into the cisterna chyli which then drains into the

thoracic lymph. Therefore, intestinal lymph may be collected by cannulation of either the mesenteric or thoracic lymph duct. However, it has been demonstrated that the collection of thoracic lymph can lead to an overestimation of the intestinal lymphatic transport of a drug because any drug absorbed via the portal blood which reaches the general circulation intact can equilibrate with peripheral lymph which then drains into the thoracic lymph.[27,28] Lymph should be collected for a sufficient length of time to enable the kinetics and full extent of the transport to be quantitated. Although it is a common practice to measure only the relative concentrations of drug in the blood and lymph as a function of time, these data are not sufficiently instructive in terms of the extent of transport to allow for a full interpretation of the data and the effect of different formulation factors. Systemic plasma levels of drug should also be determined throughout the time course of the experiment as they provide data regarding the contribution of portal blood absorption to the bioavailability of the drug. An important feature which is lacking in many lymphatic transport studies is a mass balance on the administered drug, as the combined mass of unabsorbed and metabolized drug may account for a significant component of the administered dose. These measurements, combined with estimates of absolute bioavailability, enable the contribution of lymphatic transport to the drug absorption profile and oral bioavailability to be determined.

B. LIPIDS AND BIOAVAILABILITY ENHANCEMENT

The following section describes two selected examples of drugs in which co-administered lipid produced an increase in bioavailability through different mechanisms, where the effect of food on the plasma profiles of the different drugs were qualitatively similar (late Tmax), and where the necessary animal and human data were available to substantiate the different mechanisms.

The first example considers α-tocopheryl nicotinate where co-administered lipid enhanced the bioavailability primarily through an increase in lymphatic transport, and the second example describes griseofulvin where the well known bioavailability enhancing effect of lipid was due primarily to enhanced solubilization of the drug within the intestinal milieu.

1. Tocopheryl Nicotinate: Enhancement of Lymphatic Transport

Vitamin E represents a class of eight naturally occurring phenolic compounds comprised of four tocopherols and four tocotrienols which have antioxidant activity and are thought to protect tissues against oxidation. Figure 1 depicts the structure of α-tocopherol and two commercially available derivatives. The underivatized phenolic moiety of the vitamin E compounds is susceptible to oxidation and the acetate and nicotinate ester derivatives (prodrugs) of α-tocopherol are utilized to enable formulation of pharmaceutically stable preparations. Alpha-tocopheryl nicotinate also has peripheral vasodilator activity by virtue of nicotinic acid release in the periphery of the body. Chapter 5 discusses the role of prodrugs in the lymphatic transport of some different prodrugs of vitamin E which have been studied.

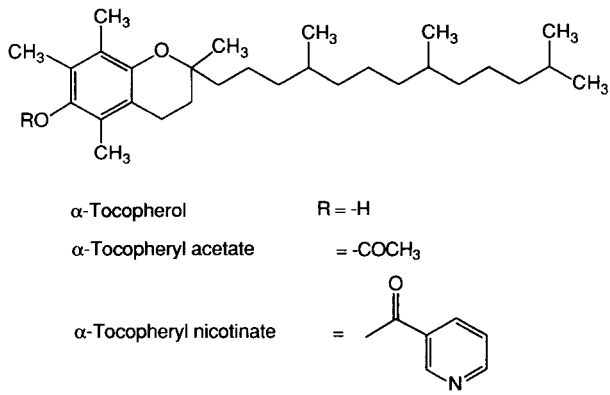


FIGURE 1. Structure of α-tocopherol and two commercially available ester derivatives.

The absorption of different vitamin E esters has been extensively studied in rats and it has been demonstrated that intestinal lymphatic transport was the major contributor to the overall bioavailability of the compounds.[29,31] Nakamura and co-workers[30] found that approximately 50% of the administered dose of a number of tocopherol esters was transported in the intestinal lymph as a combination of parent ester and unesterified α-tocopherol.

Food had a major effect on the bioavailability of α-tocopheryl nicotinate in rats, dogs, and humans.[32] Figure 2A presents the plasma levels of α-tocopheryl nicotinate after administration to human volunteers participating in a fed-fasted crossover bioavailability study. The maximum plasma concentration of α-tocopheryl nicotinate occurred at 5.5 hr post-dosing (Tmax) and the area under the plasma concentration-time profile was 28-fold greater when α-tocopheryl nicotinate was administered to fed rather than fasted subjects. Similar effects of food on the systemic plasma levels of α-tocopheryl nicotinate (fivefold increase relative to the fasted state, and an increase in Tmax to 7.5 hr) were also observed in bioavailability studies conducted in the dog.[32]

The late Tmax of the plasma profile and the overall enhancement of bioavailability of α-tocopheryl nicotinate in the presence of food are consistent with lymphatic transport of the drug which had been independently confirmed in studies conducted in lymph cannulated rats. Although the relative effects of the lipid/food on the transport of α-tocopheryl nicotinate via either the portal or lymphatic systems were not studied in the rat model, the lymphatic transport data are sufficiently compelling to conclude that the lymph is at least playing a major role. It is possible that there is a portal blood component to the transport of α-tocopheryl nicotinate to the systemic circulation although the contribution was not determined in these studies.

The effect of food and different dose forms on the bioavailability of α-tocopherol has been studied in dogs,[33] and in contrast to the α-tocopheryl nicotinate data, food had a minimal effect upon the bioavailability of α-

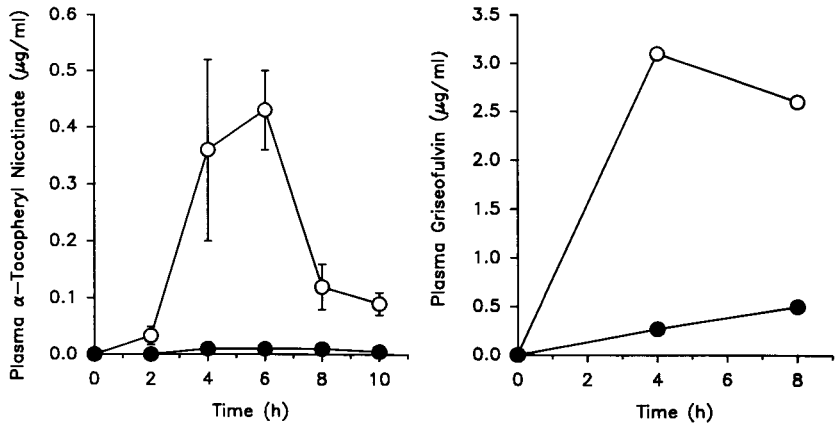


FIGURE 2. (A) Plasma concentration of α-tocopheryl nicotinate in human subjects after administration of a 600 mg dose in either the fed (○–○) or fasted state (●–●). Data are presented as Mean ± SE, n = 4. (From Hasegawa, J., Tomono, Y., Fujita, T., Sugiyama, K., and Hamamura, K., *Int. J. Clin. Pharmacol. Ther. Tox.*, 19, 216, 1981. With permission); and (B) Plasma griseofulvin concentrations after administration of a 1 g dose to human subjects in either the fasting state (●–●) or after ingestion of a high fat meal (○–○). (From Crounse, R. G., *J. Invest. Dermatol.*, 37, 529, 1961. With permission.)

tocopherol. Although higher doses of α-tocopherol were studied, the different food and vehicle effects are most likely specific for the different compounds due to differences in lipophilicity.

2. Griseofulvin: Solubilization and Dissolution

The effect of food on the bioavailability of griseofulvin is a well known example of how food can enhance the bioavailability of poorly water soluble drugs. It has been demonstrated in human subjects that administration of griseofulvin with a fatty meal produces a large increase in the measured plasma concentrations of drug relative to administration in either a fasting state or following ingestion of a low fat, high protein/carbohydrate meal.[34] A typical plasma profile of griseofulvin after administration to subjects in either the fed or fasted state is presented in Figure 2B. The peak plasma concentration of griseofulvin occurred later in the fed state and the area under the plasma concentration-time profile was greater in the fed rather than the fasted state. This plasma profile is qualitatively similar to that observed for the effect of food on the bioavailability of α-tocopheryl nicotinate; however, lymphatic transport of griseofulvin is not responsible for the increased bioavailability. Studies in lymph cannulated rats in which griseofulvin was administered as an emulsion have shown that the relative lymph/plasma concentrations of griseofulvin were approximately unity throughout the duration of the experiment.[35] These data indicate that the appearance of griseofulvin in lymph was due to redistribution from plasma and therefore lymphatic transport does not

contribute significantly to the intestinal absorption of the drug. This example demonstrates the caution that needs to be applied when interpreting plasma level data from food effect studies as the basis for improved bioavailability can potentially occur through a number of different mechanisms.

The reported mechanisms involved in the enhancement of griseofulvin bioavailability include an increased rate of dissolution of the drug due to the presence of lipid[36,37] and increased solubilization of griseofulvin due to the presence of bile salt micelles.[38] These experiments have used indirect techniques such as plasma drug concentration or urinary excretion as the means for assessing absorption of griseofulvin. A direct technique which is finding wide application for the study of drug absorption mechanisms in humans is intestinal intubation. Palma and co-workers[39] have recently studied the absorption of griseofulvin using an intestinal perfusion technique in human subjects. The drug was perfused into the duodenum dissolved in a nutrient solution which contained 27% lipid, corresponding to that of a normal meal. Drug absorption was studied as a function of lipid load and bile salt concentration present in the intestine. It was determined that bile salts were necessary for adequate solubilization of griseofulvin within the intestinal lumen and that the lipid did not directly enhance the rate of drug absorption, but was involved in the overall promotion of bioavailability. The lack of an effect of lipid on the rate of absorption may have been a result of the inability of the duodenally administered lipid to exert an effect upon gastric emptying which would otherwise regulate the rate of appearance of the drug in the duodenum. Nevertheless, these data indicate that the enhancement of griseofulvin bioavailability when administered with food is related to a combination of enhanced dissolution and solubilization.

III. LIPOPROTEIN BIOSYNTHESIS AND LYMPHATIC DRUG TRANSPORT

The intestinal lymphatic transport of lipophilic drugs is associated with the formation and transport of lipoproteins by the enterocyte which are formed in response to the absorption of lipid digestion products. Triglyceride lipid is first digested within the intestinal lumen, the digestion products are absorbed by the enterocyte, and specific lipoproteins are formed and secreted into the intestinal lymphatics. The following section describes the processes involved in the formation of lipoproteins and their role in intestinal lymphatic drug transport of lipophilic molecules.

A. SIZE AND COMPOSITION OF THE MAJOR INTESTINAL LIPOPROTEINS

Chylomicrons and very low density lipoproteins (VLDL) are the major classes of triacylglycerol-based lipoproteins formed by the enterocyte. High density lipoproteins (HDL) are also synthesized in the intestine,[40,41] but they

do not play a significant role in lymphatic drug transport. Chylomicrons and VLDL vary in terms of physical size, relative composition of triglyceride, phospholipid, apoproteins and a variety of sterol components, and whether or not they are primarily formed in response to an exogenous lipid load or from endogenous sources. Intestinal lymph varies between a slightly cloudy fluid produced under fasting conditions to an opaque/milky fluid after lipid administration — the milky appearance being primarily due to the presence of chylomicrons.

Chylomicrons are triacylglycerol-rich, spherical particles which are generally within the size range of 50 to 500 nm in diameter and are formed in response to lipid ingestion. Consequently, the triacylglycerol composition of the chylomicrons predominantly reflects that of the ingested lipid, although there is always a component of endogenous lipid.[42] A typical composition of chylomicrons would comprise 86 to 92% triacylglycerol, 6 to 8% phospholipids, 2 to 4% sterols (free and esterified) and 1 to 2% protein.[43] The spherical chylomicron is composed of a nonpolar hydrophobic core containing triacylglycerol, cholesterol esters, and about one-third of the unesterified cholesterol which is surrounded by an exterior surface coating of polar phospholipid, specific apoproteins, unesterified cholesterol, and some unsaturated triglyceride.[44] There is sufficient phospholipid present to cover approximately 80% of the surface of the chylomicrons and sufficient protein to cover up to 20% of the surface.[44] The surface coating of protein and phospholipid thereby stabilizes the hydrophobic core of the chylomicron in the otherwise aqueous environment of the lymphatic fluid. The size, rate of formation, and chemical composition of the chylomicron varies with the composition and quantity of ingested lipid.

In contrast, the smaller VLDL (20 to 50 nm) are present in mesenteric lymph under fasting conditions and, due to their smaller size, the triacylglycerol composition of the VLDL is decreased to approximately 50%. Therefore, simply from a relative volume standpoint, the VLDL would represent a less efficient transport process for dietary-based lipid relative to the larger chylomicrons. It is thought that the lipid composition of the VLDL in fasting lymph is derived primarily from endogenous intestinal and biliary sources[45] and that the VLDL play only a minor role in the translocation of exogenous lipids.[46]

B. INTESTINAL LIPOPROTEINS AND DRUG TRANSPORT

The intestinal lymphatic transport of lipophilic drugs and xenobiotics is dependent upon association/incorporation of the lipophilic compound with lipoproteins formed by the enterocyte. Table 2 presents selected data from different drug transport studies in which lymph was collected from rats and fractionated into either chylomicron, VLDL, or other lipoprotein fractions. The administered lipid vehicles were long chain fatty acid triglycerides which promote the formation of chylomicrons and other associated lipoproteins by the enterocyte. The common feature of the lymphatic transport of the different

TABLE 2
Relative Distribution of Some Lipophilic Drugs, Vitamins, and Xenobiotics Between Different Lipoprotein Fractions in Lymph

Dose and compound administered	Vehicle	Distribution of drug between lipoproteins	Ref.
3H-Retinol (600/6000 I.U.)	Soybean oil	98% chylomicrons 0.8–9% VLDL/HDL	28
Vitamin D_3	Soybean oil	95% chylomicrons	28
DDT (0.2–60 mg)	Olive oil	87–92% chylomicrons	51
Coenzyme Q-10 (0.6 mg/kg)	Hydrogenated castor oil	81% chylomicrons 10% VLDL	49
Hexadecane	Safflower oil	99% chylomicrons	50
Octadecane		98% chylomicrons	
DDT		97% chylomicrons	

compounds listed in Table 2 was that the majority of the transported drug was associated with the chylomicron fraction of the lymph. Smaller quantities of transported drug were associated primarily with the VLDL fraction, as well as other lymph fractions. These data clearly indicate the dependence of lymphatic drug transport of lipophilic compounds on the formation and transport of chylomicrons. Other indirect evidence includes transport studies in which there has been a direct dependence of lymphatic drug transport on the quantity of lipid present in the lymph,[15,47] or in which lymphatic drug transport was dependent upon the administration of the drug in a lipid vehicle which would promote lipoprotein formation.[4,11,24,48]

It is important to note that there will be a quantity of lymphatically transported drug not associated with the lipoproteins, which reflects the effective partitioning of the drug between the lipoproteins and the infranatant fraction of the lymph. However, as the log P of lymphatically transported compounds is generally greater than 5 to 6, the amount of drug not associated with the lipoproteins is probably beyond the specificity of the separation procedures and the sensitivity of the assays. Furthermore, it is likely that the unassociated quantity of these lipophilic drugs is bound to the numerous proteins which are present in the lymph.

It is generally assumed that lymphatically transported lipophilic drugs/xenobiotics are "dissolved" within the nonpolar triacylglycerol core of the chylomicron. For example, cholesterol esters and cholesterol are distributed between the interior and surface of the chylomicron with the distribution appearing to reflect the relative lipophilicity of the ester and parent compound, respectively.[44] Sieber and co-workers[51] demonstrated that at least 90% of DDT transported via chylomicrons was associated with the triacylglycerol core lipids, and Vost and Maclean[50] have demonstrated near quantitative transport of lipophilic aryl and alkyl hydrocarbons in the triacylglycerol phase of chylomicrons. However, it appears that in specific cases the triacylglycerol solu-

bility analogy may be an over-simplification as interactions between the surface components of the chylomicron and transported lipophilic compounds have been suggested.[52]

It is interesting to speculate that the relative interaction of lymphatically transported drugs with either the interior or surface components of the chylomicron may be dictated by the physicochemical characteristics of the solute, the fatty acid composition of the triacylglycerol fraction of the chylomicron core, and other factors which may include molecular size and the relative distribution of specific hydrophobic and hydrophilic functional groups within the solute molecule.

C. FORMATION OF LIPOPROTEINS BY THE ENTEROCYTE

The presence of intestinal lipoproteins in the lymph is the result of a number of highly orchestrated and complex biochemically-mediated processes. These include digestion of lipid within the lumen of the small intestine, absorption of fatty acids, monoglycerides, and other essential compounds into the enterocyte, resynthesis of the fatty acids into triacylglycerols, and synthesis of phospholipids and specific apoproteins for addition to the developing surface of the triglyceride droplets within the enterocyte. Following the formation and "assembly" of lipoproteins by the enterocyte, reverse pinocytosis of the lipoprotein into the interstitium and eventual passage into the lacteals present within the intestinal villi occurs. What makes this phenomena all the more amazing is that ingested lipid can appear within intestinal lymph as completed lipoproteins within 13 to 14 min.[53] From a capacity standpoint, the small intestine in the human can digest and transport lipid in significantly greater excess than the 100 to 200 g of triglyceride ingested on a daily basis in the typical Western adult diet.[54]

The following sections are designed to provide a brief overview of the sequence of events and processes involved in fat transport and the means by which they are related to the lymphatic transport of lipophilic drugs. For greater detail, the reader is referred to a number of excellent reviews.[43,55-61]

1. Preduodenal and Duodenal Lipid Digestion

The process of lipid digestion represents the initial step in the sequence of events leading to the formation of lipoproteins by the enterocyte as it supplies the source of long chain fatty acids and monoglycerides necessary for the resynthesis of lipoprotein triglyceride. Lipophilic drugs, associated with administered lipids which subsequently undergo digestion, are solubilized in bile salt mixed micelles along with the lipid digestion prior to absorption.

The characteristics, role, and the specificities of the many different enzymes involved in the digestion of lipids within the gastrointestinal tract have been described in Chapter 2. As described by Carey and co-workers,[56] gastrointestinal lipid digestion consists of three sequential processes: (i) dispersion of bulk fat globules into a high surface area, finely-divided emulsion, (ii) enzy-

matic hydrolysis of fatty acid esters (primarily triglycerides) at the surface of the oil/water interface, and (iii) the dispersion of the lipid digestion products into an absorbable form.

The stomach plays an important role in initiating lipid digestion and presents to the duodenum a milieu which facilitates the ongoing and further emulsification and digestion of the ingested lipid. Muscular contractions, normal peristalsis within the stomach, and movement of the stomach contents through the pylorus produce sufficient shear to form a "crude" emulsion. The initial hydrolysis of triglycerides is mediated via preduodenal lipases which, although species dependent, include enzymes of lingual and/or gastric origin.[62-64] Some absorption of the smaller chain length fatty acids can occur directly from the stomach.[65]

The major and quantitative hydrolysis of long chain triglycerides occurs within the upper small intestine due to the action of pancreatic lipase — an interfacial enzyme that specifically hydrolyzes the primary ester groups of triglycerides.[66,67] The final products of the lipolysis are the two corresponding fatty acids and a 2-monoglyceride. The 2-monoglyceride can undergo an intramolecular rearrangement to form the sn-1 isomer which is then susceptible to further hydrolysis. Although the ratio of sn-1 to sn-2 monoglycerides in an equilibrium mixture of monoglycerides is generally about 90:10, at physiological pH and temperatures the acyl migration rate is reasonably slow and unlikely to occur to a significant extent prior to absorption of the 2-monoglyceride.[68]

The presence of two additional pancreatic derived enzymes, co-lipase and phospholipase A_2, are necessary for effective and quantitative triglyceride lipolysis. Co-lipase (a coenzyme) is an absolute requirement for lipolytic activity of pancreatic lipase at oil/water interfaces in the presence of bile salts.[69,70] Co-lipase and phospholipase A_2 are secreted from the pancreas as a co-proenzyme and proenzyme, respectively, and each is activated in the lumen by tryptic cleavage.[71,72] Phospholipase A_2 requires the presence of calcium ions for activity and specifically catalyses the hydrolysis of the sn-2 fatty acids in a range of different phosphoglycerides (e.g., lecithin) to form the corresponding lyso-phosphoglycerides (e.g., lyso-lecithin).[73,74]

The interactions between lipase, co-lipase, amphiphilic bile salts, phospholipase A_2, calcium, and the triglyceride substrates present in the lumen are highly complex and dependent upon the relative concentrations of each agent present.[56] Carey and co-workers[56] have summarized the typical concentration ranges of the different enzymes and lipids present in the intestine during lipid digestion and absorption. Much of the data regarding the concentration-dependent interactions of these enzymes and substrates come from controlled *in vitro* studies which often provide the best avenue for investigation of lipid digestion; the caveat being that the results are highly dependent upon the chosen conditions. The following observations have been chosen to exemplify the complexity of fat digestion and are not necessarily intended to represent the *in vivo* situation. For example, it has been demonstrated that high bile salt concentra-

tions can desorb lipase from triglyceride/water interfaces and thereby significantly decrease the release rate of fatty acids. However, addition of co-lipase to the system restores the lipase activity by providing a binding or "anchoring" site for the lipase at the interface.[70] In other model triglyceride systems emulsified with lecithin, the addition of phospholipase A_2 has been demonstrated to decrease the lag time for appearance of fatty acids and to increase the hydrolysis rate of the triglycerides. This effect is postulated to occur via hydrolysis of the emulsifier and thereby enhanced turnover of the oil/water interface and facilitated access to triglyceride for the lipase/co-lipase system.[75] Phospholipids play an important role in aiding the solubilization of the lipid digestion products. However, their metabolism and interaction with the other factors involved in the digestion and absorption of lipids remains to be clarified.[76] The role of calcium ions and bile salts on the lipase-catalyzed hydrolysis of lecithin-stabilized triglyceride emulsions has been recently studied *in vitro* and a mechanistic model of the lipolysis process has been proposed.[77]

As the process of lipase-catalyzed triglyceride hydrolysis is complex due to the numerous concentration-dependent interactions which occur with co-lipase, bile salts, phospholipids, and triglyceride substrates,[78] it is unlikely that the mechanism of lipase activity is related to a single pathway.[79]

2. Solubilization and Absorption of Lipid Digestion Products

The micellar solubilization of fatty acids, as a prerequisite to intestinal absorption, is dependent upon the hydrocarbon chain length.[65] Short and medium chain fatty acids are not incorporated into bile salt micelles to significant extents due to their reasonably good water solubility. However, the very low water solubility of long chain fatty acids is greatly enhanced by incorporation in the mixed micelles.[80,81] As a general guide, the intrinsic water solubility of fatty acids is decreased by a factor of approximately 2.3 for each additional methylene group,[65] although the effects of chain length on the solubility in model-mixed micellar systems is reported to approach a fourfold reduction for each additional methylene group.[82] As a general guide, there is an inverse relationship between the aqueous solubility of the fatty acids and the degree of unsaturation.

As indicated, the involvement and role of phospholipids (and the lyso-product) in the mixed micelle system for the solubilization of fatty acids and monoglycerides is poorly defined.[76] Likely effects range between an increase in the size of the bile salt mixed micelle (and potentially a decreased rate of diffusion) and an increase in the solubilizing properties of the micelle toward the fatty acids and monoglycerides.

In the bulk aqueous phase, fatty acids and monoglycerides exist as monomers in solution which are in equilibrium with the solubilized form present in the mixed micelles. It is likely that only the monomeric fatty acids and monoglycerides contribute to enterocyte uptake as the mixed micelle is unlikely to be absorbed intact. The mixed micelle containing the solubilized

digestion products must then diffuse from the bulk medium and traverse the unstirred water layer (UWL) which is present over the surface of the small intestine and represents a significant diffusional barrier. Having traversed the UWL, the mixed micelle appears to dissociate into the constituent lipid digestion components which are then passively absorbed by the enterocyte.[59,61,83–86]

The above description of lipid solubilization and transport is the so-called classical approach which stems from the early investigations of Hoffmann and Borgström[81,87] and others[88,91] which characterized the digestion process as forming a "two-phase" system — one phase being composed of oil droplets (0.2 to 5 μm) dispersed in an aqueous medium and the other being a bile salt mixed micellar phase to support the removal of the lipid digestion products.

It now appears that the existence of these two discrete phases may be a function of the methodology employed in the handling of the samples.[56,90,91] Light microscopy and associated studies of fat digestion conducted by Patton, Carey, and co-workers[56,92–94] have demonstrated the sequential appearance of a solid crystalline phase on the outside of the oil droplet consisting primarily of calcium salts of the fatty acids followed by the production of a viscous isotropic, or liquid crystalline phase, composed predominantly of monoglyceride and protonated fatty acids.

The disparity between these observations and the classical Hofmann-Borgström approach have apparently been clarified through phase equilibrium studies of simplified model systems. It is likely that the aqueous phase of the early studies actually represented a combination of liquid crystalline and micellar phases.[56,95] It is important to note that the contributions of the different phases present within the lipid digestion milieu are highly dependent upon the relative concentrations of the bile salts and other components in the system.[96]

Using the light microscope, Patton and co-workers[92] were able to follow the direct transfer and passage of either colored or fluorescent lipophilic markers from the triglyceride lipid phase to the enzyme product phase during fat digestion. They suggested the existence of a continuous hydrophobic domain between the triglyceride phase and the fatty acid and monoglyceride digestion phase which remained intact during lipolysis. These digestion products were then solubilized by the bile salt micelles, leading to transfer and subsequent absorption by the enterocyte of the digestion products. These observations regarding the flow of lipid digestion products have been termed a hydrophobic continuum or domain and the concept is significant in terms of the transfer and eventual absorption of hydrophobic compounds (drugs or xenobiotics) which are associated with ingested triglyceride. This factor is described in more detail in Section IV B.1. Although the role of the hydrophobic domain and its occurrence during fat digestion *in vivo* is difficult to quantitate, as the studies have been conducted under highly controlled *in vitro* conditions, the concept provides a useful description for the transfer of hydrophobic compounds from a dissolved state in triglyceride into bile salt mixed

micelles containing the lipid digestion products. Patton[94] has also suggested that the hydrophobic continuum may even allow for direct transfer of the digestion products into the enterocyte, although the mechanisms by which the lipid digestion products gain entry into the cell remain to be determined.

Recent and very comprehensive studies of the physical chemistry of dietary and biliary lipids during digestion and absorption under simulated and *in vivo* conditions have demonstrated that unilamellar vesicles of variable size may co-exist with bile salt mixed micelles during lipid digestion.[97,98] It has been proposed that unilamellar vesicles most likely supply the mixed lipid digestion products to the micellar phase in a readily dissolvable form. Figure 3 depicts the putative role of the unilamellar vesicles, simple and mixed micelles in lipid digestion, and the solubilization of digestion products.

In summary, lipid digestion produces two primary phases with the actual composition of the micellar phase being determined by the concentrations of the different components present.[56,96–98] The hydrophobic domain/continuum may play a role during the lipolysis of the triglyceride oil droplets and the transfer of digestion products to the micellar phases. However, the passage of digestion products and other nonpolar molecules into the enterocyte, the potential interaction with bile salt micelles, the passage and interaction of the micelles with the unstirred water layer (including the mucin and glycocalyx), and the mechanism by which the lipid digestion products are absorbed by the enterocyte are all yet to be fully elucidated.[43,56,59,65,96]

3. Synthesis of Triglyceride by the Enterocyte

The fatty acids and monoglycerides absorbed by the enterocyte are first visible morphologically as triglyceride droplets present within the smooth endoplasmic reticulum. These triglyceride droplets which form the core of the chylomicrons are sequentially processed through the cell, as described later, and are secreted into the lymph. The specificity and functional nature of the different enzymes involved in these pathways have been described in Chapter 2. Figure 4 depicts the range of biochemical reactions involved with the transport of absorbed lipid as lipoproteins secreted into the lymph from the enterocyte which occur in response to absorbed lipid digestion products.

It has been known for a long time that the chain length of the absorbed fatty acid dictates its relative transport by either the intestinal lymphatics as resynthesized triglyceride, or via the portal blood as free form fatty acid.[99,102] Fatty acids of chain length greater than 12 carbons are predominantly transported by the intestinal lymph, whereas short and medium chain fatty acids are transported by the portal blood. There is a reasonably well founded biochemical basis for these chain length effects which includes the specificity of fatty acid-CoA synthetase for fatty acids of chain length greater than 12 carbons and the relative affinity of the intracellular fatty acid binding protein (FABP) for long chain unsaturated fatty acids.[65,103–105] Additional factors which affect the relative partitioning of fatty acids between the portal blood and the lymph

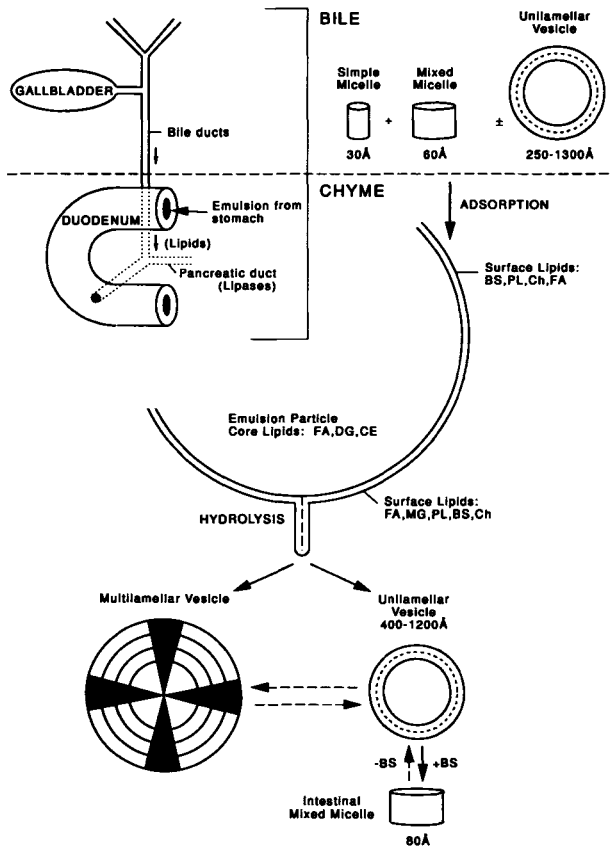


FIGURE 3. Proposed model for the sequential steps in fat digestion. Biliary lipids (present as either simple or mixed micelles or vesicles) and lipase and co-lipase adsorb to the surface of the crude emulsion particles entering the duodenum from the stomach. The core lipids of the crude emulsion are triglyceride (TG), diglyceride (DG), and cholesterol esters (CE). During lipolysis, the fatty acid (FA) and monoglyceride (MG) lipid digestion products are primarily located at the emulsion surface. As the size of the emulsion droplet decreases with continuing lipolysis, the build up of FA, MG, and phospholipids (PL) at the surface continues and portions of the surface coat "pinch-off" to form a variety of structures which are dependent upon the concentration of bile salts (BS) present. It was postulated that unilamellar vesicles originate from liquid lamellar crystals that form at the surface of the emulsion-water interface. The interconversion between the vesicles and mixed micelles is favored by the presences of unsaturated BS micelles. (From Hernell, O., Staggers, J. E., Carey, M. C., *Biochemistry*, 29, 2041, 1990. With permission.)

include the degree of unsaturation and the rate of fatty acid absorption.[106–108] Realistically, fatty acid transport via the lymph or the portal blood is unlikely to be as "clear-cut" as the above description, as there are reports of lymphatic transport of medium chain triglycerides via chylomicrons and of the portal blood transport of long chain fatty acids.[106,109–112] It is important to note that absorbed long chain fatty acid lipid digestion products are not exclusively

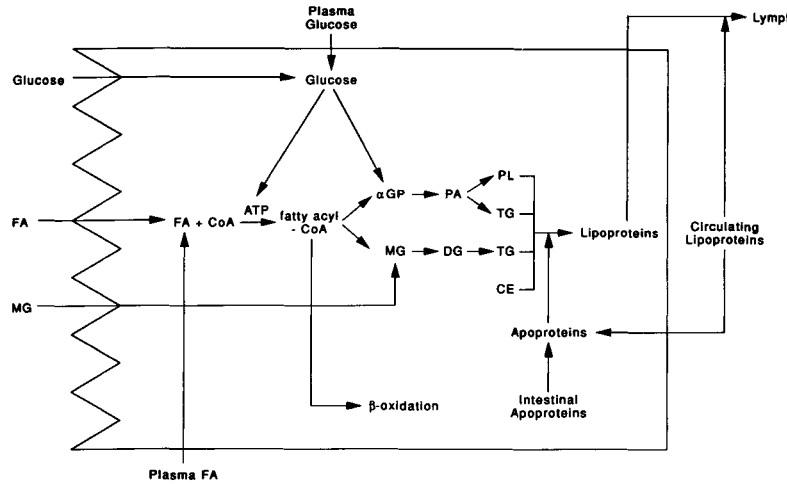


FIGURE 4. Schematic representation of the intracellular events associated with lipid transport by the enterocyte. Fatty acids (FA) derived from the lumen and plasma are activated to fatty acyl-CoA. The required ATP is predominantly derived from glucose metabolism. Fatty acyl-CoA is then esterified with either (i) α-glycerophosphate (α-GP) to form phosphatidic acid (PA) and then either triglyceride (TG) of phospholipid (PL) via the phosphatidic acid pathway, or (ii) with monoglyceride (MG) to form TG via the monoglyceride pathway. Resynthesized TG together with PL, cholesterol ester (CE) and apoproteins then form lipoproteins which are secreted via reverse pinocytosis into the lymph. During fat absorption, chylomicrons are the predominate lipoproteins and VLDL predominate under fasting conditions. The apoproteins are derived from intestinal sources via synthesis and some are most likely derived from the plasma by filtration. (From Shiau, Y.-F., *Am. J. Physiol.*, 240, G1, 1981. With permission.)

utilized for the resynthesis of triglyceride by the enterocyte. The absorbed fatty acids can be subjected to metabolic oxidative reactions which depend primarily upon the availability of alternate energy sources from the cell.[58,113]

There are two pathways for the formation of triglyceride by the enterocyte and they are known as either the monoacylglycerol or phosphatidic acid pathways. Although these two pathways utilize fatty acyl-CoA as the fatty acid donors, and 1,2-diglycerides are intermediates of both pathways, they function independently of each other. The monoacylglycerol pathway involves the direct acylation of absorbed exogenous 2-monoacylglycerol with suitably activated fatty acids to form the corresponding diglyceride which is then sequentially acylated to form triglyceride.[114,115] The phosphatidic acid pathway (also known as the glycerol-3-phosphate or α-glycerophosphate pathway) involves the sequential acylation of endogenous glycerol-3-phosphate with three molecules of activated fatty acid in a complex series of biochemical pathways.[116,117] Phosphatidic acid is an intermediate diglyceride product of the phosphatidic acid pathway and can serve as a substrate for the subsequent synthesis of either triglyceride or the corresponding phospholipid (Figure 4).

The activated fatty acids (fatty acyl-CoA) destined for either pathway are formed by fatty acid-CoA synthetase, and the fatty acid source can involve

both exogenous and endogenous components. The distribution of exogenous and endogenous fatty acids in the final triglyceride depends upon the lipid load, the class of the administered lipid, and the pathway primarily responsible for the resynthesis of the triglyceride.[43,58,118,119] The exogenous glycerol produced upon the complete luminal hydrolysis of triglyceride does not appear to be directly involved in the formation of the 3-glycerol-phosphate for triglyceride resynthesis.[65]

The monoacylglycerol pathway has the highest capacity, is most rapid and energy efficient, and can account for approximately 80% of chylomicron triglyceride synthesized following the administration of triglycerides which contain long chain fatty acids.[120] Triglyceride produced from this pathway is likely to have a short residence time within the cell and is generally transported from the cell via chylomicrons.[121] The data from a number of different studies using radiolabeled lipids indicate that the monoglyceride pathway is the preferred route for chylomicron triglyceride synthesis.[111,122] In contrast, triglycerides formed via the glycerol-3-phosphate pathway are most apparent following administration of either free or simple esters of fatty acids (in the absence of monoglycerides) and the chylomicrons that are produced are typically smaller in size than when triglyceride is produced via the monoglyceride pathway.[43] The mucosal cell residence time of triglyceride produced via this pathway is longer than that synthesized via the monoacylglycerol pathway.[121] Additionally, there are indications that some of the triglyceride produced through the phosphatidic acid pathway may exit the cell through pathways other than the lipoproteins.[60,122,123]

Intestinal triglyceride transport studies utilizing Pluronic L-81 (a synthetic hydrophobic surfactant which specifically interferes with the assembly of chylomicrons), or the supplemental addition of phosphatidylcholine to the lipid infusions have indicated that there appear to be different pathways for the formation and assembly of chylomicrons and VLDL by the enterocyte.[111,119,124–126] These observations have been supported by studies which have suggested that there are distinct pools of triglyceride within the enterocyte[122] and that the destiny of the lipid may be, in part, dictated by the different triglyceride synthetic pathways.[111,121,122] The different factors which determine the processing and regulation of long chain fatty acids and monoglycerides within the enterocyte and their role in the formation of triglyceride destined for chylomicrons (and other lipoproteins or destinations) are yet to be fully determined.

4. Phospholipids

Phospholipids (PL) play a major role in lipid digestion and are involved in the transport of lipoproteins by the small intestine. The role of PL in lipid digestion involves the promotion of emulsification of the ingested triglycerides which aids further digestion and the solubilization of the digestion products within the bile salt mixed micelle.

Phospholipids play an important role in the transport of dietary fat from the small intestine by stabilizing the surface properties of the lipoproteins

formed by the enterocyte. Zilversmit[44] estimated that phospholipids are present over about 80% of the chylomicron surface, thereby stabilizing its hydrophobic triglyceride core in the otherwise aqueous environment of the lymph. Phosphatidylcholine (PC) is the primary (80%) phospholipid present on the chylomicron surface, although there are significant quantities (4 to 6%) of other phospholipids which include phosphatidylethanolamine, lysophosphatidylcholine, and phosphatidic acid.[127]

The liver synthesizes PL destined for either the systemic circulation or the bile, and it appears that separate pools within the liver supply PL to either the bile or plasma.[128,129] The transport of PC into, and by, the bile requires bile salts and it appears that the presence of cholesterol is also necessary.[76,103–132] PL present within the small intestine is derived from both dietary and biliary sources. In the human, the bile supplies approximately 3 to 4 times the quantity of PL generally available from the diet, and the bile is consequently considered to play a greater role in lipid transport than the dietary derived PL.[76]

Phosphatidylcholine must be hydrolyzed prior to absorption by the enterocyte. Pancreatic phospholipase A_2 specifically hydrolyzes the fatty acid at the 2-position of PC to form lyso-PC and the corresponding fatty acid which are then available for absorption by the enterocyte.[73,74] Although there are different pathways for the utilization of the absorbed lyso-PC,[76] the major pathway within the small intestine is re-esterification with an absorbed fatty acid to form PC which is then available for addition to the surface of the developing lipoproteins (primarily VLDL and chylomicrons). The fatty acid source for the re-esterification of lyso-PC can closely reflect the composition of exogenously administered lipid.[133]

The utilization of biliary and/or dietary derived PC in the transport of lipoproteins appears to be dependent upon the relative amounts of PC from either source presented to the gut. In the extreme case, using conditions of limited PC supply in bile-diverted rats, Mansbach[134] observed that nearly all the PC present within the chylomicrons was derived from exogenous sources.

The enterocyte is also capable of *de novo* synthesis of PC and it appears that there is an inverse relationship between the amount of PC formed via the *de novo* pathway and the quantity of PC present within the lumen from exogenous and endogenous sources.[134] The *de novo* synthesis of PC is mediated via cholinephosphotransferase which utilizes CDP-choline and diglyceride as substrates. The diglycerides are produced via the α-glycerophosphate pathway which is also responsible for the formation of triglyceride by the enterocyte. The synthesis of CDP-choline appears to be the rate limiting step in the formation of PC via the *de novo* pathway.[135]

From lymphatic transport studies in bile-diverted rats, it appears that the supply of PC from the *de novo* pathway is unable to sustain the transport of high fat loads from the enterocyte and that supply of biliary or exogenous PC is necessary for the efficient transport of high fat loads.[134,136,137] Interestingly, it has been demonstrated that the lymphatic transport of triglyceride in intact rats can be increased by supplementation with exogenous PC, raising

the question as to the potential rate limiting role of PC in lymphatic lipid transport.[121,138]

As described by Tso and Scobey,[76] although the crucial role of phospholipids has been clearly demonstrated in the transport of dietary lipid, the detailed aspects of the different structural features of the phospholipids and their involvement in the sequential formation and assembly of chylomicrons (and other lipoproteins) by the enterocyte remains to be fully characterized.

5. Apoprotein Synthesis and Assembly of Chylomicrons

There is a protein component present on the surface of all lipoproteins and these protein moieties are termed apoproteins or apolipoproteins. The apoproteins play a crucial role in the synthesis and transport of chylomicrons, as lipid transport studies conducted in the presence of protein synthesis inhibitors such as acetoxycycloheximide or puromycin have demonstrated accumulation of triglyceride droplets within the enterocyte and decreased transport of chylomicrons into the lymph.[139–141] Apoproteins are also directly involved in the subsequent clearance and metabolism of the chylomicrons and other lipoproteins by the liver and within the general circulation. Although the role of the different apoproteins in the assembly and clearance of lipoproteins is beyond the scope of this chapter, the following section briefly describes the role of protein synthesis as it relates to chylomicron formation.

The relative amount (% weight) of apoprotein present on the surface of the different lipoproteins varies inversely with the size and surface area of the lipoprotein. The surface protein component of the chylomicrons is comprised of a number of different specific apoproteins. The apoproteins present in chylomicrons isolated from intestinal lymph of humans and the rat are qualitatively similar and comprise apo B (10%), apo A-IV (10%), apo E (5%), apo A-I (15 to 35%) and apo C (45 to 50%).[55] An additional apoprotein, apo A-II, is present in human chylomicrons but is only a minor component of the apoproteins in chylomicrons from the rat.[55]

It appears that the small intestine does not synthesize all the apoproteins that are present in isolated chylomicrons. Although active synthesis of apo B and the apo A proteins has been shown to accompany triglyceride resynthesis and chylomicron formation by the enterocyte,[43,55,142] it does not appear that the intestine produces significant amounts of apo E and C.[143,144] The presence of apo E and apo C in chylomicrons isolated from mesenteric lymph arises through these apoproteins becoming associated with the chylomicron subsequent to secretion by the enterocyte.[145,146] The complex involvement of the apoproteins in lipoprotein formation is further evident when it is realized that the apoproteins are in fact glycolipoproteins, and there is some controversy regarding the actual site(s) of glycosylation of the apoproteins within the enterocyte.[147]

The major role of apo B, a glycosylated protein, in the assembly and transport of chylomicrons from the enterocyte has been confirmed through studies of abetalipoproteinemia, an inherited disease characterized by an in-

ability to form lipoproteins containing apo B. The inability of these patients to synthesize apo B leads to accumulation of triglyceride droplets in the intestine and an impaired secretion of chylomicrons.[148,149] Ultrastructural investigations have shown apo B to be localized in the rough endoplasmic reticulum (RER) and to be present on triglyceride droplets in the smooth endoplasmic reticulum (SER), which indicate that the addition of the apo B occurs early in the process of chylomicron formation.[55,150]

The organelles primarily involved in the formation and secretion of chylomicrons are the endoplasmic reticulum (smooth and rough) and the golgi body complex. Although morphological studies have provided the majority of information regarding the assembly and transport of chylomicrons,[141,147,151–153] the description and details of the processes involved is still incomplete.

The following sequence of assembly and transport is suggested from these morphological studies. Triglyceride is synthesized from the passively-absorbed fatty acids and monoglycerides in the structures of the endoplasmic reticulum (ER) which contains the requisite enzymes and substrates. Morphological studies conducted after lipid administration have readily demonstrated droplets of triglyceride in the cisternae of the SER (Figure 5). The endoplasmic reticulum network is a complex intracellular network of tubular structures which are able to promote the enzymatic processes and also channel the products of the biosynthetic reactions to other subcellular organelles. The synthesis of triglyceride in the ER places it in an ideal position for the subsequent addition of the apoproteins formed by the RER and the phospholipids which stabilize the surface of the triglyceride. The RER synthesizes at least some of the apoproteins present in the final chylomicron, and these are added to the developing surface of the triglyceride. It appears that the RER can initially supply a pool of apoproteins in anticipation of triglyceride synthesis. The lipid-rich products of these events occurring in the SER and RER are termed "nascent chylomicrons" and represent an almost complete secretory product (Figure 6).

The classical description of the final assembly and excretion of chylomicrons from the enterocyte involves the obligatory movement of the nascent chylomicrons through the channels of the endoplasmic reticulum to the Golgi apparatus/complex in preparation for the so-called "final assembly and secretion". It is believed that the final processing of the nascent chylomicrons in the Golgi complex involves the association of lipid and protein moieties and/or final protein glycosylation. Morphological studies have indicated that multiple pre-chylomicron particles are present and "packaged" in Golgi derived secretory vesicles. These secretory vesicles which generally contain multiple chylomicrons are transported, potentially via the intracellular microtubule network, and undergo membrane fusion with the lateral cell plasmalemma (Figure 7). The secretion of the chylomicrons present in the secretory vesicles is mediated through bulk exocytosis, and the morphologically complete chylomi-

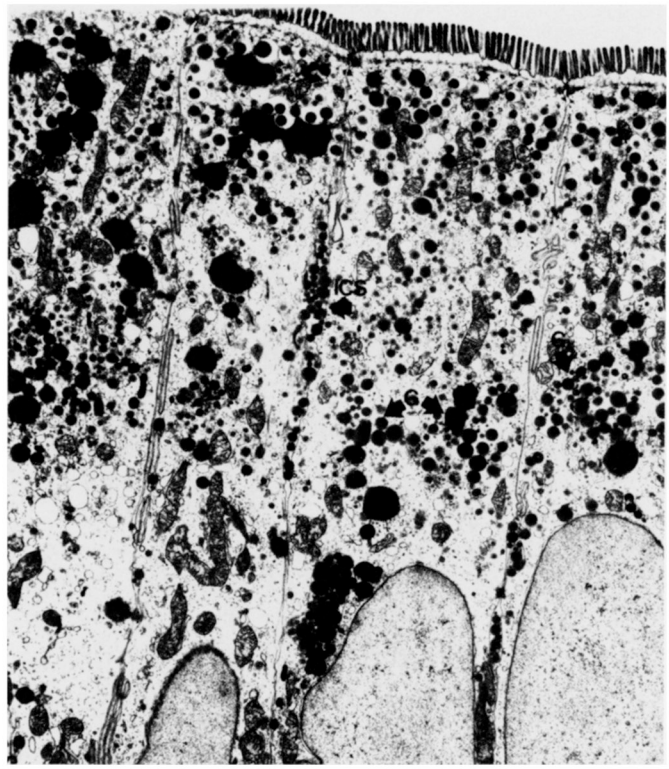


FIGURE 5. Electron micrograph (× 8,000) taken 2 hr after feeding corn oil to rats. The apical cytoplasm of the intestinal absorptive cells has become filled with osmiophilic droplets bounded by membranes of the smooth endoplasmic reticulum. The Golgi zones (G) are distended with nascent chylomicrons. The Golgi areas contain osmiophilic droplets of chylomicron size and the intercellular spaces (ICS) are filled with lipid droplets of chylomicron size (120 to 480 nm) indicating that secretion has occurred. (From Sabesin, S. M. and Frase, S., *J. Lipid. Res.*, 18, 496, 1977. With permission.)

crons enter the intercellular space. The chylomicrons then enter the lamina propria through gaps in the adjacent endothelial cells.

A recent study of intestinal lipid transport conducted in the trout has questioned the obligatory role of the Golgi apparatus prior to exocytosis of the chylomicrons from the cell.[147] The study indicated that, in addition to the classical role of Golgi apparatus in chylomicron transport, triglyceride-rich particles were also directly transported from the endoplasmic reticulum via secretory vesicles which then fused with the cell plasmalemma leading to exocytosis of the lipoprotein into the intercellular space (i.e., the Golgi apparatus was bypassed). The relevance of these findings and the role of lipoprotein transport via pathways other than the Golgi apparatus remain to be determined.

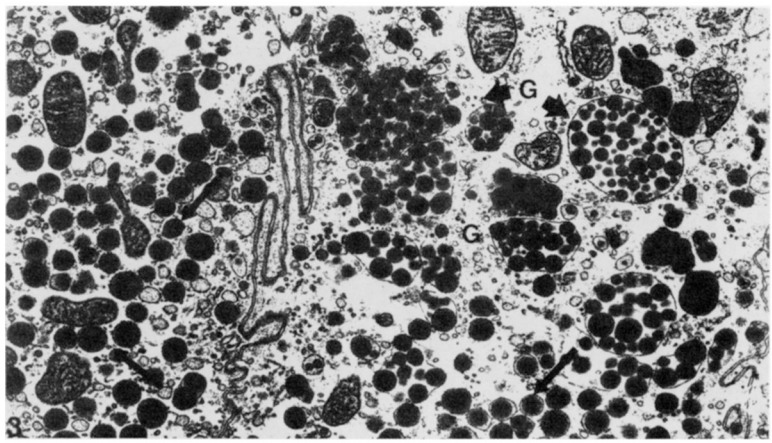


FIGURE 6. Electron micrograph (× 13,377) of a cell filled with innumerable fat droplets located within the vesiculated channels of the smooth endoplasmic reticulum (long arrows). A Golgi zone (G) contains many vesicles filled with nascent chylomicrons measuring 60 to 350 nm. (From Sabesin, S. M. and Frase, S., *J. Lipid Res.*, 18, 496, 1977. With permission.)

IV. INTESTINAL LYMPHATIC TRANSPORT AND LIPID VEHICLES

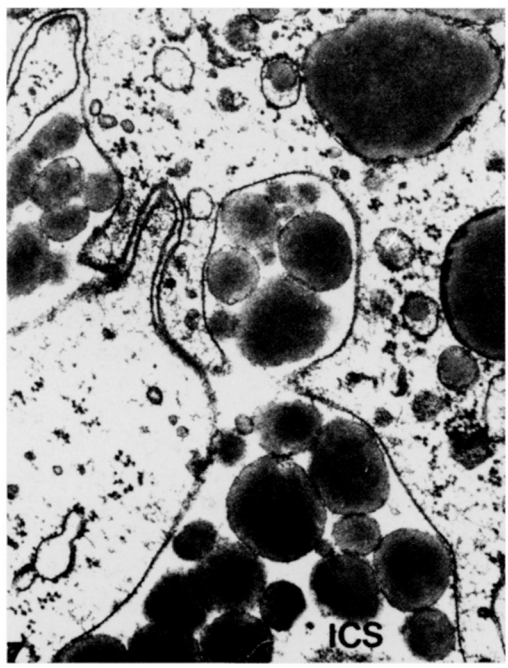
There are limited examples describing the relative effect of solid- and lipid-based formulations on the delivery of lipophilic drugs. This is probably due to the relatively small number of orally administered drugs which are primarily transported by the lymphatic system, and the fact that the majority of reported data describe the effect of lipid vehicles on bioavailability. This section deals exclusively with the role that different lipids and formulations play in determining the extent of intestinal lymphatic transport of lipophilic drugs.

A. REQUIREMENT FOR CO-ADMINISTERED LIPID

One compound for which a comparison has been made between lipid and solid formulations is testosterone undecanoate, a prodrug of testosterone used for the treatment of-androgen deficiency syndromes in males. The oral administration of testosterone is limited by nearly quantitative pre-systemic clearance.[154] The undecanoate ester prodrug was developed as an orally active form of testosterone in which the prodrug was transported via the lymph to the systemic circulation, thereby avoiding the significant first pass effect. The effect of formulation on the oral bioavailability of testosterone has been studied in humans.[155,156]

Figure 8 depicts the mean increment in bioavailable testosterone (determined from plasma AUC values after allowing for the endogenous levels present in the male subjects) after administration of either a micronized testos-

A



B

FIGURE 7. (A) A secretory vesicle (SV) containing nascent chylomicrons has fused (arrow) with the lateral plasmalemma (× 44,160), and (B) the exocytosis of chylomicrons is evident as the nascent chylomicrons present in the secretory vesicle are similar in size and morphology to the chylomicrons already present in the intercellular space (ICS). (From Sabesin, S. M. and Frase, S., *J. Lipid Res.*, 18, 496, 1977. With permission.)

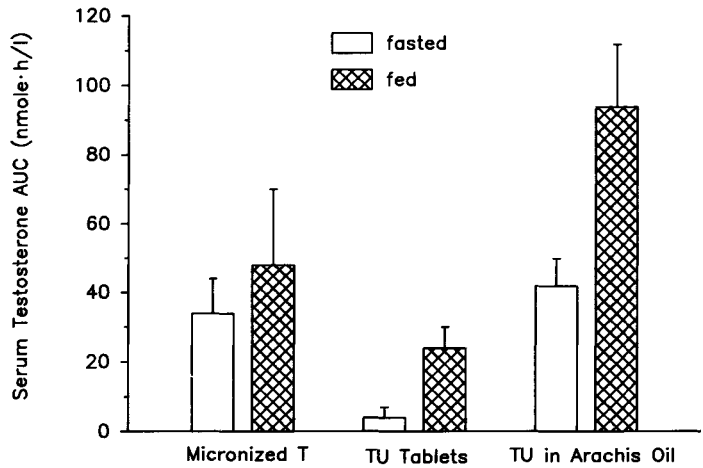


FIGURE 8. Mean increment in bioavailable testosterone expressed as area under the serum testosterone concentration-time profiles (AUC^{0-6}, nmol.hr/l). The forms of testosterone (T) administered were, (A) 100 mg micronized T, (B) 100 mg testosterone undecanoate (TU) as tablets equivalent to 63 mg T, and (C) 100 mg TU dissolved in arachis oil. The different formulations were administered in either the fed of fasted states. (From Frey, H., Aakvaag, A., Saanum, D., and Falch, J., *Eur. J. Clin. Pharmacol.*, 16, 345, 1979. With permission.)

terone (T) formulation, a tablet formulation of testosterone undecanoate (TU), or a lipid-based formulation of testosterone undecanoate.[155] The bioavailability study, which was parallel in design, was also conducted in the fed and fasted states.

There was a significant and consistent increase in serum testosterone levels after administration of the micronized T preparation and the magnitude of the plasma levels were marginally increased when the formulation was administered with food. However, from a therapeutic standpoint, the large doses of the solid T formulation which would be necessary to produce an increase in the plasma levels of circulating T in male subjects are inappropriate for long-term androgen replacement therapy due to potential toxicity concerns.[155] There were marked formulation effects on the bioavailability of T when administered as the TU prodrug. The bioavailability of TU administered as a tablet formulation in the fasted state was less than that of T when administered in micronized form, although administration of TU as a tablet in the presence of food restored the bioavailability to similar levels as observed following administration of micronized T. The increased bioavailability of T from the solid formulation of the TU prodrug in the presence of food probably reflected both an increased dissolution and solubilization of the drug relative to the fasted state and the formation of chylomicrons by the enterocyte in response to the lipid component of the food. It has been independently

demonstrated that TU is trans-ported almost exclusively via the intestinal lymphatics in association with the lipoproteins.[157,158]

Administration of TU in a lipid vehicle (5 ml arachis oil, a triglyceride which principally contains $C_{18:1}$ fatty acids) produced an eightfold to tenfold enhancement relative to the tablet formulation when administered in the fasted state. This is presumably related to a combination of (i) the formation of lipoproteins by the enterocyte in response to the administered lipid thereby facilitating the lymphatic transport of TU, and (ii) the presentation of solubilized TU rather than a tablet formulation which may have impaired dissolution characteristics. Administration of TU solubilized in the arachis oil formulation in the presence of food produced a further twofold to threefold enhancement of the bioavailability relative to the fasted state. This further enhancement is likely to be a function of delayed gastric emptying and increased chylomicron transport due to an increased lipid load (food consisted of a 1986 kJ fatty breakfast, comprising 59% fat), and possibly reflects an increased solubilization of the drug within the intestinal tract due to the digestion of the ingested meal. It is interesting to speculate that the 5 ml of arachis oil administered in the fasting state was not sufficient to initiate the full cascade of events which lead to the slowing of gastric emptying, efficient digestion, solubilization of lipid digestion products, secretion of pancreatic and biliary fluids, and the synthesis of lipoproteins by the enterocyte. A search of the literature did not reveal any significant data regarding the quantity of lipid (in a dose-response manner) which would need to be administered to humans in order to initiate such events.

Although these data demonstrate a significant increase in the effective bioavailability of T following oral administration of the undecanoate ester, it is important to note that the absolute bioavailability of TU, when studied in female subjects, was still only in the order of 3 to 6% of the administered dose.[156] In the rat, it has been demonstrated that the low oral bioavailability of TU does not appear to be related to metabolism of TU by the liver, as the prodrug was not transported via the portal blood and only low concentrations of T were found in the portal blood after intraduodenal administration of the ester.[158] This is consistent with other studies where portal blood transport of lipophilic prodrugs was not generally observed.[159,160] These studies indicate that TU probably undergoes extensive pre-hepatic clearance within the enterocyte and/or the intestinal lumen and that only the nonmetabolized portion of the administered dose is transferred via the intestinal lymphatics to the systemic circulation.

A further example of the relative effect of solid- or lipid-based dose forms on the bioavailability of a lipophilic drug where intestinal lymphatic transport is the major determinate of bioavailability is the hypolipidemic agent, LK-903 (1-O-[*p*-(myristyloxy)-α-methylcinnamoyl]-glycerol).[13,48] This compound is the corresponding glycerol analog of the free acid form, LK-A (*p*-(myristyloxy)-

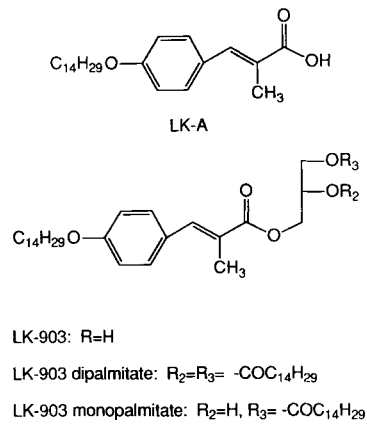


FIGURE 9. Structures of LK-A, LK-903, and the mono- and di-palmitate esters of LK-903.

α-methyl cinnamic acid) and the structure of the two compounds are shown in Figure 9. The mono- and di-acyl fatty acid analogs of LK-903 are observed *in vivo* and, because of their increased lipophilicity, are the major contributors to the lymphatic transport (and subsequent bioavailability) of the compound. The role of the different acylated forms, their lymphatic transport, and the relative conversion to either LK-903 or LK-A are described in Chapter 5.

The major fraction of LK-A derived material in the systemic circulation is primarily due to the formation and lymphatic transport of the di-acylated form of LK-903 which can be progressively cleaved to yield the mono-acylated form, LK-903 and LK-A.[13] As would be expected, the bioavailability of LK-A and LK-903 are highly dependent upon the characteristics of the administered dose form. The relative bioavailability of LK-A or LK-903 from different formulations studied in the dog are depicted in Figure 10. The bioavailability of either LK-A or LK-903 increased significantly in the presence of co-administered lipid relative to administration of a solid tablet formulation. The requirement for the administered lipid enables the formation of lipoproteins by the enterocyte and hence transport of the di-acylated LK-903 analog via the lymphatics. These observations are also consistent with a significant increase in the bioavailability of LK-903 when administered in the fed as opposed to the fasted state in dogs.[48] The further enhancement of the bioavailability of either the free acid or glycerol derivative when administered in a lecithin-containing formulation is noteworthy. It is likely that the further increase in plasma levels of LK-A and LK-903 with the lecithin-containing formulations is due to an overall increase in the quantity of drug reaching the enterocyte due to enhanced solubilization and a subsequent increase in the degree of lymphatic transport.[13,160]

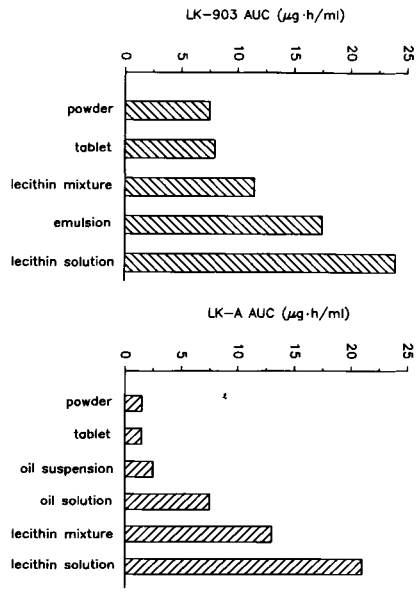


FIGURE 10. The effects of different formulations on the oral bioavailability of LK-903 and LK-A when administered to beagle dogs. The lecithin mixture was a 1:16 mix of LK-903 (or analog) with egg yolk lecithin, and the lecithin solution was a 16-fold aqueous dilution of the mixture. The emulsion was a 20% Tween 80 solubilized system, and the oil solution was a solubilized formulation containing 62% linoleic acid. (From Mizobe, M., Matsuda, S., Yoneyama, T., and Kohno, K., *Chem. Pharm. Bull.*, 31, 2424, 1983. With permission.)

Disseminated adrenocorticol cancer or pituitary-dependent Cushing's syndrome have been clinically treated with o,p′-DDD, a compound structurally related to DDT.[161-163] Sieber[14] has demonstrated in a rat model that intestinal lymphatic transport plays a major role in the absorption of orally administered o,p′-DDD and that the compound is transported in the lymph in association with chylomicrons. The apparent bioavailability of o,p′-DDD when administered in different vehicles has been studied in human subjects.[164] Although the study was not a crossover design (due to the long elimination half-life of the drug), the bioavailability of the compound was significantly enhanced when administered in the presence of lipid. For example, in studies conducted in fasted patients, the plasma levels were significantly elevated when the compound was administered as an arachis oil emulsion or in combination with either chocolate or milk powder relative to ingestion as a solid formulation. The improved bioavailability of o,p′-DDD when administered in the presence of the digestible lipid is most likely a manifestation of a combined enhancement of lymphatic transport and overall promotion of bioavailability. These results are further supported by a recent study of the bioavailability of o,p′-DDD when administered as different formulations in a crossover study per-

TABLE 3
Bioavailability Parameters When o,p′-DDD Was Administered in Different Vehicles to Dogs in Either the Fed or Fasted State[a]

Formulation[b]	AUC^{0-12h} (mg.hr/l)	C_{max} (μg/ml)	T_{max} (hr)
Solid tablet (fasting)	2.4 ± 1.06	0.4 ± 0.1	4.5 ± 0.3
Emulsion[c] (fasting)	40.1 ± 3.15	11.0 ± 0.6	2.8 ± 0.2
Ground tablets dispersed in lipid (fed)	63.9 ± 5.82	15.4 ± 2.5	3.3 ± 0.6

[a] Mean ± SE (n = 6 dogs).
[b] All doses were 50 mg/kg.
[c] Emulsion contained maize oil emulsified with gum arabic.

Taken from Watson, A. D. R., Rijnberk, A., and Moolenaar, A. J., *Res. Vet. Sci.*, 43, 160, 1987. With permission.

formed in dogs.[165] The study was also performed in the fed and fasted states. Table 3 describes the effect of food and different formulations on the plasma levels of o,p′-DDD when administered to dogs. The bioavailability was increased 20- to 30-fold when administered either in the presence of food or when administered as an emulsion formulation. As was the case with the human study, these drug-plasma concentration data do not enable delineation of the factors involved in the enhanced bioavailability of o,p′-DDD, although they most likely reflect a combination of enhanced drug availability within the intestinal lumen, absorption via the portal blood, and enhancement of lymphatic transport.

B. ROLE OF LIPID DIGESTION AND DRUG UPTAKE

1. Solubilization

The processes by which the digestion products of triglycerides are solubilized, transported, and absorbed by the enterocyte are not fully understood due to the complex concentration-dependent interactions between the lipid digestion products, bile salts, cholesterol, and phospholipids present within the intestinal lumen during lipid digestion. Recent studies by Carey and co-workers[97,98] have added significantly to previous knowledge of the physical-chemical properties of dietary and biliary lipids during intestinal digestion and absorption, by determining the phase behavior and aggregation states of lipids undergoing digestion and absorption. The pertinent details of these studies have been described in Section III C.

In vitro studies conducted by Patton, Carey and co-workers[25,56,92] have demonstrated the apparent flow of lipid digestion products and hydrophobic drug markers from the surface of triglyceride droplets undergoing lipolysis to the lipid digestion product phases. Patton[94] has suggested that fat digestion

provides a hydrophobic domain or continuum for the transfer of digested lipid from the surface of the emulsified triglyceride in the intestinal lumen via progression from a viscous isotropic phase, to the bile salt mixed micellar system which carries the products of lipolysis (and other highly lipophilic compounds which may be present), to the surface of the absorbing enterocyte. The importance of the bile salt mixed micelle and other liquid crystalline vesicles/phases for promoting the solubilization of lipophilic drugs and xenobiotics is clearly demonstrated by the markedly enhanced solubility of hydrophobic compounds in the presence of lipid digestion products. Studies of polycyclic aromatic hydrocarbons,[166] nonpolar lipids, and cholesterol[167] have indicated that the capacity of the bile salt micelle to solubilize the hydrophobic compounds is greatly enhanced when the bile salt micelle contains an appropriate quantity of the fatty acid and monoglyceride lipid digestion products. The increased solubility of exogenous lipophilic compounds is thought to be a function of increased size of the micelle due to the swelling nature of the amphipathic lipid digestion products and potentially to specific interactions with the components present in the micelle.[58] The chain length of the fatty acid and monoglyceride appears also to affect the magnitude of the increased solubilization. For example, studies have demonstrated that the solubilization of α-tocopherol in mixed bile salt micelles was increased by a factor of between threefold and sevenfold when the lipid component comprised long chain fatty acid lipids as opposed to medium chain length fatty acids.[168]

It is common practice to speculate about the effects of different lipid vehicles on the observed lymphatic transport of lipophilic drugs. However, it is not a straightforward task to evaluate the intrinsic effects that different formulations and lipid components have upon the absorption process of the drug and the lipid digestion products by the enterocyte. Ideally, evaluation of different lipid-based formulations on the absorption component of lymphatic transport would include analysis of (i) the efficiency of digestion of different lipid vehicles, and (ii) the properties of the lipid bile salt mixed micelles and other pertinent digestive phases as a function of the administered formulation.

An example of the insight that can be gained from studies of this type is the determination of the basis for the reduced lymphatic transport of glycerol tristearate relative to glycerol trioleate. Bergstedt and co-workers,[169] in a comprehensive study which assessed the extent of lipid hydrolysis and the distribution of lipid within the mucosal cells, demonstrated that the lower lymphatic transport of the glycerol tristearate was due to a combination of poorer digestibility of the tristearate lipid and a less extensive incorporation of resynthesized triglyceride into chylomicrons. Unfortunately, analyses of this magnitude are often impractical for routine application to drug transport studies and consequently, the majority of the lipid effect data on lymphatic drug transport often ignores the important role of the pre-absorptive process as a prerequisite to lymphatic transport.

2. Enterocyte Related Metabolism of Lipophilic Drugs

Although the liver is generally considered the major detoxifying organ within the vertebrate body in terms of drug and xenobiotic metabolism,[170] it is also recognized that gut wall metabolism can play a major role in limiting the bioavailability of orally administered drugs.[171] The enterocyte is involved in a range of Phase I, hydrolytic, and Phase II drug metabolism reactions as well as the potential formation of a variety of different lipid-based drug conjugates.[171–174] The *in vivo* study of drug metabolism by the enterocyte is not a straightforward task, as the studies often require *in situ* experimental systems with the sampling of a variety of biological fluids (e.g., lymph, portal blood, and bile) as a function of time and the quantitation of drug and metabolite levels. This is especially the case when studying the metabolism of highly lipophilic drugs (and prodrugs) which are absorbed into the enterocyte in association with the lipid digestion products, and are potentially involved in the process of lipoprotein formation and assembly. Recent advances which have been made in developing stable and viable cell culture systems of intestinal cells, which are capable of producing and secreting lipoproteins,[175–178] offer significant potential for the study of the metabolism (and targeting) of drugs which are associated with the products of lipid digestion by virtue of their high lipophilicity.

Patton and co-workers[15,25] have studied the intestinal absorption, lymphatic transport, and metabolism of benzo(a)pyrene (BP), a carcinogenic polycyclic aromatic hydrocarbon which gains access to the systemic circulation via intestinal lymphatic transport. The studies were conducted in a conscious rat model in which both the mesenteric lymph duct and the common bile duct were cannulated and lymphatic transport was promoted by administering BP dissolved in olive oil.[15,25] The lymphatic transport of BP, lipid, and the biliary excretion of metabolized BP were measured as a function of time throughout the experiment.

During the first 2 hr after administration, the lymphatically transported BP was associated with the chylomicrons in the lymph; however, the concentration of BP in the chylomicron lipid was less than 1% of the administered concentration in the olive oil solution. Although the formation and transport of chylomicrons continued at elevated levels for at least 7 hr after administration of BP dissolved in olive oil, the lymphatic transport of BP in the lymph dropped dramatically after the initial 1 to 2 hr. However, during the 2- to 7-hr period post-dosing, radiolabeled compound(s) were apparent in the bile at levels much greater than initially transported in the lymph despite the continued formation and secretion of chylomicrons by the enterocyte. The biliary associated radiolabeled material was presumably derived from the absorption via the portal blood of polar BP metabolites which were formed by the enterocyte after the initial absorption of BP and subsequent secretion in the bile. In these studies, the cumulative 24 hr recovery of radiolabeled compounds in lymph, bile and urine amounted to approximately 20% of the administered 0.1 mg dose of BP with intestinal lymphatic transport accounting for only 2%

of the administered dose. Approximately 80% of the radiolabeled compound which was absorbed from the intestinal tract of the rat was excreted in the bile as BP metabolites. In further studies of BP absorption and transport which were conducted in a killifish model, it was also determined that greater than 80% of the recovered radioactivity following BP administration was found in the gall bladder of the fish. These data indicate that the majority of the BP absorbed by the enterocyte was metabolized to more polar compounds which were then transported to the liver via the portal blood. Furthermore, it appears that the enterocyte is able to rapidly differentiate between the triglyceride lipid to be transported via the lymphatics and the highly lipophilic BP which is diverted via the portal vein to the liver as a result of the formation of more polar metabolites.

The combined results from these studies conducted in the rat[15] and the killifish[25] suggest that the administered BP remains associated with the lipid digestion products during micellar solubilization, transport to the enterocyte, and movement through the cytosolic structures of the cell. However, the endoplasmic reticulum, which is the site of triglyceride biosysnthesis within the enterocyte destined for packaging into the chylomicrons, is also a major source of the enzymes which are capable of metabolizing BP and other polycylic aromatic hydrocarbons.[179,180] Consequently, the formation of polar BP metabolites by the enzymes present in the endoplasmic reticulum leads to their dissociation from the resynthesized triglyceride, probably due to their increased hydrophilicity, and then being transported to the liver via the portal blood.

Another example of enterocyte mediated metabolism of a highly lipophilic compound is naftifine. This compound is an antimycotic agent which is susceptible to a very high first pass effect due primarily to cytochrome P-450-dependent oxidation and subsequent conjugation.[10] The lymphatic transport of naftifine has been studied in a rat model as a potential means of bypassing the otherwise high first pass effect after oral administration. It was determined that approximately 2% of the administered dose (10 mg/kg) was transported in the lymph with the remainder of the absorbed compound appearing in the portal blood as more polar metabolites. Grimus and Schuster[10] suggested that the low lymphatic transport of the highly lipophilic naftifine was a result of metabolism of the drug after absorption by the enterocyte, with the subsequent transport of the polar naftifine metabolites via the portal blood. In an analogous manner to the work described for BP, these workers suggested that based upon the distribution of the enzymes in the enterocyte most likely responsible for the metabolism of naftifine, it was probable that the drug was metabolized at the endoplasmic reticulum in competition with the formation of triglyceride for the synthesis of chylomicrons.

Based upon the current understanding of lipid digestion and solubilization, it is likely that highly lipophilic drugs/xenobiotics are solubilized within the intestinal lumen in conjunction with the lipid digestion products, are trans-

ported to the enterocyte in association with the lipid digestion products, and are subsequently absorbed. Unless the enterocyte was able to effectively clear (metabolize) highly lipophilic drugs/xenobiotics from the absorbed and resynthesized lipid, the lymphatic transport of the compounds would otherwise be assured based solely on the grounds of their high lipophilicity. Although the site within the enterocyte which is responsible for the separation of highly lipophilic compounds from absorbed lipid digestion products is largely unknown, the endoplasmic reticulum is generally implicated due to the range of drug metabolizing enzymes present within the structure. The potential for drug metabolism within the enterocyte is most likely one of the major reasons (excluding poor absorption) that the intestinal lymphatic transport of lipophilic drugs is not more common.

Before significant advances can be made in the area of intestinal lymphatic transport, a better understanding of the potential for the separation of drugs/xenobiotics from absorbed lipid and the development of appropriate strategies for minimizing the extent of metabolism is required. It is likely that isolated cell culture techniques will contribute significantly to this endeavor. This approach holds special significance for the success of developing lipophilic prodrugs (and indeed pro-moieties) for the enhancement of intestinal lymphatic drug transport.

C. CHOICE OF CO-ADMINISTERED LIPID

The appropriate choice of co-administered lipid is a crucial determinate of the maximal intestinal lymphatic transport which can be achieved for lipophilic drugs. This section will briefly describe, with examples, the effects of different lipids on the extent of either their own lymphatic transport or that of co-administered lipophilic compounds. Caution needs to be exercised when examining the following section, and the data described within the cited references, in terms of rigidly defining trends for the effects of different lipids on lymphatic transport because (i) there is a paucity of data describing the lymphatic transport of both drugs and the co-administered lipid vehicle, and (ii) the differences observed may be a function of either luminal-based factors (e.g., differences in administered formulation, emulsification, lipid digestion, solubilization) and/or enterocyte-based factors (e.g., differences in lipid processing, lymph transport versus portal blood absorption).

1. Fatty Acid Chain Length and Class of Administered Lipid

The effect of fatty acid chain length on the relative transport as free form fatty acids by the portal blood or as resynthesized triglycerides in the lymph has been described in Section III C.3. It was indicated that fatty acids with chain length less than 12 carbons were predominantly transported in the portal blood with the longer chain fatty acids being transported in the lymph as re-esterified triglyceride. The basis for the relative effects of fatty acid chain length on the consequent triglyceride lymphatic transport was rationalized in biochemical

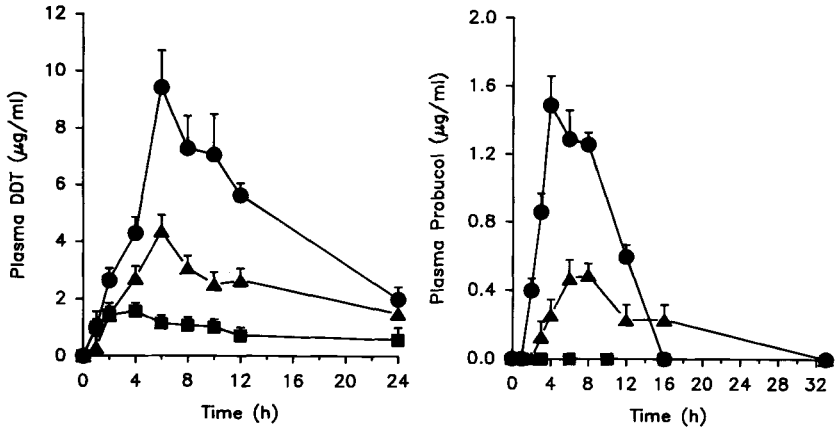


FIGURE 11. Plasma concentration-time profiles of DDT and probucol after oral administration to rats in different lipid vehicles. Key: ●-●, arachis oil; ▲-▲, miglyol 812; ■-■, liquid paraffin. (From Palin, K. J., Wilson, C. G., Davis, S. S., and Phillips, A. J., *J. Pharm. Pharmacol.*, 34, 710, 1982; and Palin, K. J. and Wilson, C. G., *J. Pharm. Pharmacol.*, 36, 641, 1984. With permission.)

terms, although it was pointed out that the demarcation between lymphatic and portal blood transport was less "clear cut" than is often assumed.

Palin and co-workers[11,24] have demonstrated the effect of fatty acid chain length on the lymphatic transport of two highly lipophilic compounds (DDT, a pesticide; and probucol, a hypocholesterolaemic agent) where the bioavailability and resulting plasma levels of either compound are primarily determined by intestinal lymphatic transport. The mean plasma levels of DDT and probucol, which were determined in rats after oral administration of either compound dissolved in different lipids, are presented in Figure 11. The different lipids which were studied were arachis oil (a triglyceride containing primarily $C_{18:1}$ fatty acids), Miglyol 812 (a triglyceride containing primarily C_8-C_{10} saturated fatty acids), and liquid paraffin, which is a poorly absorbed nondigestible mineral oil. The trends in the lipid vehicle effects on lymphatic transport were consistent between compounds, with the highest plasma levels being achieved after administration of the arachis oil vehicle and the lowest plasma levels of either compound being evident after administration in the nondigested mineral oil. The plasma levels of either compound were intermediate following administration in the medium chain triglyceride vehicle.

As lymphatic transport is the major contributor to the plasma levels of either compound, the following explanation is consistent with the observed vehicle effects. Digestion of arachis oil within the small intestine liberates long chain fatty acids which, once absorbed and resynthesized by the enterocyte to triglyceride, will promote chylomicron formation and consequently the lymphatic transport of the co-administered DDT or probucol. The digestion of the Miglyol 812 triglyceride vehicle will produce medium chain fatty acids which are most likely absorbed via the portal blood and do not promote the formation

of chylomicrons by the enterocyte. The plasma levels of either compound observed after administration in the Miglyol vehicle are most likely a reflection of portal vein transport. The absorption of either compound into the portal blood may be augmented by the digestion of the triglyceride and liberation of medium chain fatty acids into the intestinal milieu. It is possible that the medium chain glycerides have an inherent propensity for the promotion of absorption of some drugs.[31,181,182] The low plasma levels of DDT and probucol evident after administration in the paraffin oil vehicle are consistent with an inability to form chylomicrons.

Similar fatty acid chain length effects on the absorption and lymphatic transport of some other xenobiotics have been observed. For example, the effect of fatty acid chain length on the intestinal absorption of 7,12-dimethylbenz(a)anthracene in the rat has been studied following administration in olive oil, safflower oil, or a medium chain triglyceride.[183] The presence of bile significantly enhanced the absorption in the presence of long chain fatty acid triglycerides although it did not affect the absorption after administration in a medium chain triglyceride vehicle. In the presence of bile, the absorption of 7,12-dimethylbenz(a)anthracene was significantly greater when administered in the long chain fatty acid vehicle as opposed to the medium chain triglyceride. Similar fatty acid chain length effects were observed when studying the absorption of 3-methylcholanthrene in mice.[184] The lymphatic transport of 3-methylcholanthrene was greater when administered in either olive or sesame oil relative to administration in trioctanoin.[185]

The intestinal lymphatic transport of testosterone undecanoate in a mesenteric lymph duct cannulated conscious rat model has been studied after administration in a number of different lipid vehicles of varying fatty acid chain length.[158] The rank order profiles of lymphatic transport exhibited a similar trend as observed with DDT and probucol where the systemic plasma levels were quantitated. The lymphatic transport was maximal when administered in an oleic acid vehicle (0.5% of administered TU dose) and decreased by a factor of approximately five when administered in a medium chain triglyceride vehicle. As the basis for the lymphatic transport of the highly lipophilic prodrug is the formation of chylomicrons, the observed transport profiles were consistent with the known absorption and transport of long versus medium chain fatty acids.

The class of lipid (triglyceride or free form fatty acid) containing long chain fatty acids has been shown to affect the rate and extent of intestinal lymphatic transport of DDT in a rat model.[8] DDT (2 mg) was administered intraduodenally to anesthetized rats in either 200 μl of oleic acid or an equivalent triglyceride (arachis oil) vehicle. The cumulative transport of DDT is depicted in Figure 12 as a function of the administered lipid vehicle. The extent of transport of DDT from the oleic acid vehicle was approximately 30% of the administered dose. In comparison, the triglyceride vehicle produced a substantially lower lymphatic transport of DDT, and the lag time for transport was longer with this vehicle.

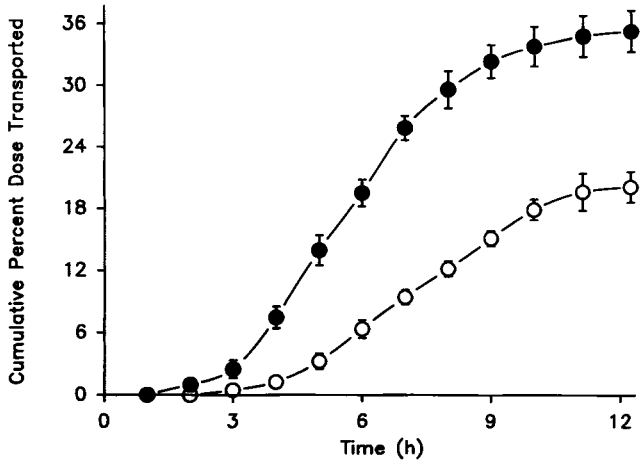


FIGURE 12. Cumulative recovery (Mean ± SE) of DDT in intestinal lymph after intraduodenal administration in either oleic acid (●–●) or arachis oil (○–○) to rats. The dose of DDT was 2 mg and the administered volume of lipid was 200 µl. (From Charman, W.N. and Stella, V. J., *Int. J. Pharmaceut.*, 33, 165, 1986. With permission.)

In the same study, the quantities of lipid transported in the lymph over the 12-hr sampling period, estimated using a simple turbidimetric procedure, were similar between the different vehicles. Therefore, the different quantities of DDT transported were not a function of different extents of lipid transport per se. The longer lag time associated with administration of the triglyceride vehicle reflected the requirement for pre-absorptive hydrolysis to the constituent fatty acids and monoglyceride, whereas the more rapid transport of DDT after administration in oleic acid was consistent with more rapid resynthesis of triglyceride and chylomicron transport. In this study, the apparent loading of DDT per unit of lymphatic lipid (representing the lipoproteins) was assessed and it was found that the concentration of DDT per unit weight of lipid was reflected in the different extents of transport between the vehicles (i.e., the fatty acid vehicle was able to maintain a higher loading of DDT per unit of lymphatic lipid than did the triglyceride vehicle). These observations were consistent with the more rapid transit of lipid and DDT from the lumen into the enterocyte when administered in the oleic acid vehicle which did not require digestion.

2. Saturated and Unsaturated Fatty Acids

The absorption and lymphatic transport of unsaturated and saturated long chain fatty acids has been the subject of study for many years, and more recently with renewed interest following the observation that diets rich in saturated fats raise serum cholesterol, whereas diets rich in polyunsaturated fatty acids are associated with a reduction in serum cholesterol.[43]

The following data have been chosen to exemplify the apparent trends which have been observed in terms of fatty acid composition and the resulting

lipid transport. It is important to remain cognizant of the difficulties in extrapolating between trends in transport data of administered lipids of varying degrees of unsaturation, and the likely effect that the different lipids would have upon the transport of a co-administered lipophilic drug.

In general, the absorption and transport of saturated lipid is less efficient than that for unsaturated lipids although the extent of transport is dependent upon the chain length of the administered long chain fatty acid lipid and the extent of unsaturation. Although lipid transport data are highly method-dependent, estimates range between 10 to 50% less efficient transport for saturated fats relative to "corresponding" unsaturated fats.[169,186,187] The composition and size of intestinal lipoproteins[188] are affected by the nature of the saturated and unsaturated lipid administered, and the structural features of the lipoproteins reveal marked changes which are dependent upon the composition of the administered lipid.[189] Various studies have indicated that absorption and transport of lipids with increasing degrees of unsaturation produced an increase in the size of lymph lipoproteins.[186,188] However, there is clearly a limit to the increased size of the lipoproteins and extent of lipid transport observed with increasing degrees of unsaturation. For example, the lymphatic transport of triglyceride in a rat model after administration as either olive oil (containing primarily $C_{18:1}$) or a polyunsaturated fish oil (containing primarily $C_{20:5(n-3)}$ and $C_{22:6(n-3)}$) was similar in terms of absorption and mass transport, and the composition of the lymph triglycerides reflected the fatty acids in the administered lipids.[190]

Studies of the intestinal lymphatic transport of testosterone undecanoate in a conscious rat model revealed that the lymphatic transport of the prodrug after administration in oleic acid was approximately double that achieved when administered in the polyunsaturated MaxEPA lipid.[158] However, the lymphatic transport of triglyceride from an arachis oil vehicle was similar to that acheived with the MaxEPA lipid.[190] Similarly, it has been observed that the lymphatic transport of some of the lipid soluble vitamins is decreased when administered with polyunsaturated lipids relative to administration in lipids with a lower content of polyunsaturated fatty acids.[12] The means by which these apparently divergent lymphatic drug transport data relate to the differences in apparent lymph lipid transport are unknown. Notwithstanding the difficulties of comparing data from different studies, it is interesting to speculate that an important factor, besides the mass of lipid transported in response to lipid administration, is the rate of processing of the lipid and the relative affinity that a drug (solute) has for resynthesized triglyceride within the enterocyte and the intestinal lipoproteins. Lymphatic transport studies which address these issues are likely to clarify the otherwise difficult problem of identifying and rationalizing trends in lymphatic drug transport as a function of the administered vehicle.

The rate and extent of formation of LDL, VLDL, and chylomicrons in the mesenteric lymph of anesthetized rats has been assessed following the intraduodenal administration of either arachis oil, oleic ($C_{18:1}$), linoleic ($C_{18:2}$),

or linolenic acid ($C_{18:3}$).[191] For the C_{18} fatty acids, the lag time for the appearance of chylomicrons was significantly reduced with increasing degrees of unsaturation and probably reflects an increased rate of absorption. The arachis oil vehicle produced the longest lag time for the appearance of chylomicrons in the lymph and is consistent with the need for pre-absorptive hydrolysis of the triglyceride to the corresponding fatty acids and monoglyceride. The transport of chylomicrons in the lymph reached a steady state after approximately 3 hr, and the rate of transport was dependent upon the composition of the administered lipid vehicle. The rank order trends of chylomicron transport after administration of the different fatty acids was linoleic > linolenic > oleic acid. Cheema and co-workers[191] suggested that the different rates of chylomicron transport reflected different biochemical pathways and rates of enzymatic reaction within the mucosal cell for incorporation of the lipids into chylomicrons. The transport profiles may also reflect a differential effect between absorption via the portal blood or the resynthesis of triglyceride destined for incorporation into lipoproteins.

The intestinal absorption of two isomeric $C_{18:1}$ unsaturated fatty acids has been studied in a rat model where it was determined that there was up to a threefold difference in the lymphatic transport of triglyceride lipid.[192] The isomeric fatty acids studied were 9-*cis* octadecenoic acid (oleic acid) and 9-*trans*-octadecenoic acid (elaidic acid), respectively, and the transport profiles were compared to a parallel group of rats which received a similar dose of palmitic acid, a $C_{16:0}$ saturated fatty acid. The lymphatic recovery of oleic acid as lipoprotein triglyceride was 43 to 50% of the administered dose compared with a recovery of between 10 to 17% for either the elaidic or palmitic acids. The number of chylomicrons in the lymph and the rate of triglyceride appearance in the lymph was greatest after the administration of oleic acid. Additionally, oleic acid was preferentially incorporated into lymph triglyceride when compared with elaidic acid, and it appeared that the two lipids were processed differently by the enterocyte. The lower lymphatic transport of palmitic acid relative to oleic acid was rationalized in terms of (i) decreased affinity for the fatty acid binding protein (FABP) which has greater affinity for unsaturated fatty acids, and (ii) likely utilization in the lower capacity glycerophosphate pathway which serves both triglyceride and phospholipid synthesis. These results indicate the structural and isomeric specificity of the biochemical processes within the enterocyte involved in the resynthesis of triglyceride.

A recent study of the intestinal absorption of menhaden and rapeseed oils has demonstrated that the cellular and lymphatic appearance of fatty acids from the digestion of methyl and ethyl esters of long chain fatty acids was approximately 50% less than from the corresponding triglycerides.[193] It was suggested that the slower transport of the fatty acids when administered as an alkyl ester may be due to the lower efficiency of the glycerophosphate pathways required for the resynthesis of triglyceride in the absence of monoglyceride otherwise produced through the digestion of the corresponding triglyceride.

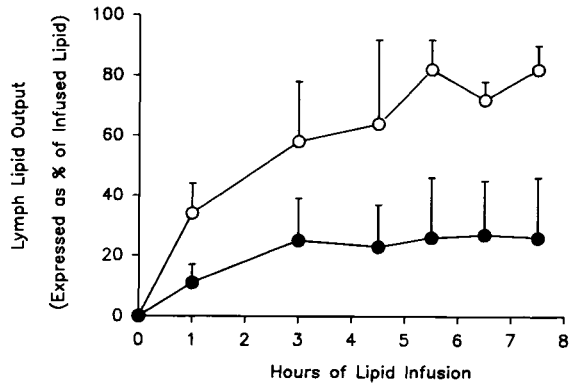


FIGURE 13. Lymph radioactive lipid transport expressed as a percentage of hourly infused radioactive triglyceride lipid infused. The rats (n = 6) were infused with either glycerol trioleate (○-○) or glycerol tristearate (●-●) and the data are presented as mean ± SE. (From Bergstedt, S. E., Hayashi, H., Kritchevsky, D., and Tso, P., *Am. J. Physiol.*, 259, G386, 1990. With permission.)

Bergstedt and co-workers[169] have studied the digestion, uptake, and lymphatic transport of either glycerol trioleate ($C_{18:1}$) or glycerol tristearate ($C_{18:0}$) under steady state conditions in rats. Figure 13 depicts the lymphatic lipid transport, expressed as a percentage of the infused lipid per hour, when rats were infused with either glycerol trioleate (TO) or glycerol tristearate (TS). The lymph lipid output in the rats which received TO showed a gradual increase and reached a steady state value of approximately 75% by the 8th hr of the infusion. In contrast, the steady state lymph lipid output during administration of TS demonstrated a much smaller increase and reached a plateau of approximately 25% between the 3rd and 8th hr of the infusion. A mass balance was performed on the infused lipid and it was found that the luminal recovery of radioactive TS was approximately 43% of the infused lipid as opposed to about 6% for the administered radioactive TO. It was determined that the majority of the radiolabel associated with the TS remaining in the intestine was intact triglyceride indicative of incomplete digestion. The studies suggested that the resynthesis of absorbed fatty acid into triglyceride by the enterocyte was equally efficient for either the $C_{18:0}$ or $C_{18:1}$ fatty acids. However, the stearate-enriched triglyceride was not as well incorporated (or packaged) into the triglyceride-rich lipoproteins as the oleic acid-enriched triglycerides. The design of these steady state studies is particularly noteworthy as they enabled a mass balance to be performed upon the administered lipid, and thereby, determination of the intrinsic factors responsible for the differences in lipid transport. Study protocols comprising this format will find application in pharmaceutical investigations of lymphatic drug transport when combined with simultaneous monitoring of portal blood absorption of drug and/or metabolite.

In summary, it is not clear how the trends in the lymphatic transport of different lipids would affect the lymphatic transport of a co-administered lipophilic compound. The protocols and methodologies which have been applied to the study of intestinal lipid transport have significant application in the assessment of intestinal lymphatic lipid and drug transport studies.

3. Volume of Administered Lipid

A common methodological variable in the assessment of intestinal lymphatic transport is the volume of the co-administered lipid vehicle.[26] In rats, the volume of lipid administered often varies between 0.05 and 1 ml, and on a bodyweight conversion basis the 50 µl volume is equivalent to approximately 10 ml for a 70 kg human. However, without knowledge of relative scaling factors in terms of gastric emptying, biliary and pancreatic secretions, efficiency of digestion, and lipid transport, it is unreasonable to extrapolate between the rat and man.

The effect of different dose volumes on the lymphatic transport of DDT[8] or benzo(a)pyrene (BP)[15] has been studied in the rat. Although the design of the respective studies was different in terms of the assessment of drug absorption and transport, both studies indicated that the extent of lymphatic transport was not dramatically affected by the volume of the administered lipid in the range studied. In the case of DDT, the volumes administered were 50 or 200 µl of either arachis oil, oleic acid, or a 2:1 oleic acid/monoolein mixture, and in the BP study the administered volumes were either 50 or 500 µmoles (corresponding to approximately 50 or 500 µl) of olive oil.

The time-dependent appearance of radiolabeled BP in the lymph was similar irrespective of the administered volume (approximately 2% of the administered dose). As described in Section IV B.2, the enterocyte appears to be able to discriminate between the resynthesized triglyceride and the absorbed BP during transit through the cell, and consequently it is difficult to speculate as to the basis for the similar extents of transport. When the concentration of BP in lymph lipid was studied 1 hr post-dosing, it was determined that the BP concentration was approximately 0.08 and 0.4% of the initial concentrations in the 50 and 500 µl olive oil, respectively, indicating that the enterocyte efficiently cleared BP from the administered lipid.

In the study of volume effects on DDT transport, the lymphatic transport was similar for the triglyceride or oleic acid vehicles in terms of the extent of transport (approximately 20 or 30% of the administered dose, respectively) although the kinetics of transport were different.[15] The lag time for appearance of DDT in the lymph after administration in the oleic acid/monoolein mixture was decreased with the lower volume of administered lipid. There was a corresponding increase in the apparent lipid load of DDT per unit of lymph lipid when administered in the lower volume of lipid, with the final concentration of DDT per volume of lymph lipid being approximately 0.5

to 2% by weight, indicating the relatively efficient lymphatic transport of DDT.

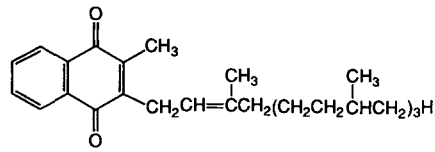
The basis for the lack of effect of different lipid volumes studied on lymphatic transport of these two compounds is uncertain. It is likely that an appropriate explanation would involve assessment of relative transfer and transport kinetic processes which include lipid digestion, solubilization, portal blood absorption, and lymphatic transport when considered in the context of lipoprotein biosynthesis.

D. ADDITIONAL COMPOUNDS TRANSPORTED VIA THE INTESTINAL LYMPHATICS

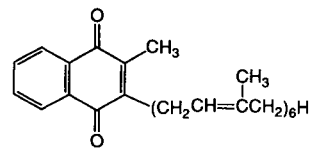
1. Lipid Soluble Vitamins

The lipid soluble vitamins are comprised of vitamins A, D, E and K — each of which are representative of a family of compounds with varying degrees of activity. The structure of the primary vitamin A, D, E and K derivatives are depicted in Figure 14. The lipid soluble vitamins are poorly soluble in water and rely primarily upon on intestinal lymphatic transport for efficient absorption and transport to the systemic circulation. Vitamin D is an exception as it can be synthesized endogenously via UV-light induced cleavage of 7-dehydrocholesterol. In general, the absorption mechanisms of the different lipid soluble vitamins are not well defined, although they are dependent upon chylomicron-based transport and hence require administration of appropriate lipids to facilitate digestion, solubilization within the lumen, and lipoprotein formation by the enterocyte.[194] Of the different vitamins, some are ingested as either alkyl (vitamin E) or fatty acid esters (vitamin A) which are initially cleaved within the intestinal lumen, and the unesterified vitamin is then absorbed. As a general guide, the major hydrolysis of the different esters of vitamin A, D, and E occurs when the esters are solubilized within the mixed micelles, although it has been demonstrated for α-tocopheryl acetate that further hydrolysis can also occur within the brush border region of the mucosal cells as well as intracellularly.[195] In particular cases, the absorbed vitamin is then reesterified by the enterocyte with absorbed long chain fatty acids.

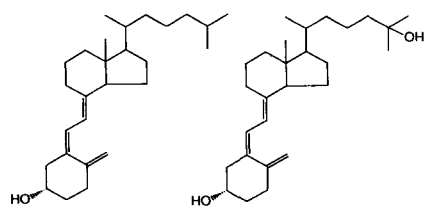
The absorption of retinol from the lumen at physiological concentrations occurs via a saturable carrier-mediated passive process which can be modified by the addition of bile salts and fatty acids.[196] However, the absorption process of retinol is dependent upon the administered dose.[194] Retinol is esterified in the enterocytes by acyl-CoA:retinol acyltransferase[197] to form primarily the palmitate ester which is transported exclusively via the chylomicrons. The relative transport of retinol in the portal blood and the intestinal lymph has been studied as a function of a number of different co-administered lipid and bile salt components.[198] The lymphatic transport of retinol decreased progressively upon the separate addition of oleic, linoleic, or arachidonic acid to the intestinal infusate but was increased upon the addition of medium chain triglycerides. The absorption of retinol by the enterocyte is apparently decreased by the addition of these unsaturated fatty acids to the perfusate.[196] However, when the relative lymphatic or portal blood transport of retinol was estimated,[198] it appeared that the decreased lymphatic transport of retinol upon addition of the



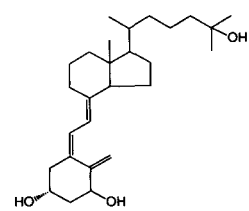
Vitamin K_1



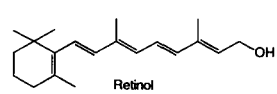
Vitamin K_2



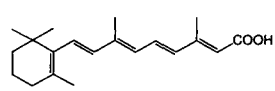
Vitamin D_3 25-OH-D_3



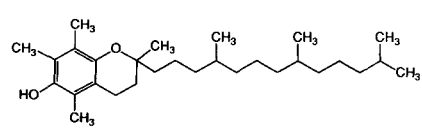
1, 25-$(OH)_2$-D_3



Retinol



Retinoic acid



α-Tocopherol

FIGURE 14. Structures of the some of the different forms of the lipid soluble vitamins.

TABLE 4
Lymphatic Transport of [^{3}H] Activity After Infusion of Either [^{3}H]-Retinol or [^{3}H]-Retinyl Palmitate

Infused formulation[a]	Percent lymphatic recovery[b]
Micellar formulation	
Retinol	49.4 ± 3.8
Retinyl palmitate	58.0 ± 8.2
Retinyl palmitate + THL[c]	17.4 ± 8.7
Emulsified formulation	
Retinol	18.0 ± 6.0
Retinol + THL	15.7 ± 2.1
Retinyl palmitate	47.8 ± 7.8
Retinyl palmitate + THL	5.0 ± 2.4

[a] Formulations were infused for a 12-hr period.
[b] Data are presented as mean ± S.D. recovery in 24-hr lymph.
[c] THL (tetrahydrolipstatin), inhibitor of lipase activity (10^{-4} M).

Taken from Fernandez, E. and Borgström, B., *Lipids*, 25, 549, 1990. With permission.

unsaturated fatty acids was accompanied by an increased transport via the portal blood as estimated by fistulation of bile.

A recent study has compared the relative lymphatic transport of retinol and retinyl palmitate when administered to thoracic lymph duct and bile duct cannulated rats as emulsion or micellar formulations.[199] The emulsified formulation consisted of triolein, phosphatidylcholine, and bile salts, whereas the micellar system consisted of a 2:1 mixture of oleic acid and 1-monoolein and bile salts. The micellar system presented the lipid components in a readily absorbable form, whereas the retinyl palmitate would have required hydrolysis prior to absorption. The lymphatic transport of radiolabeled retinol (present as retinyl ester) when administered as either retinol or retinyl palmitate as a function of the different formulations is presented in Table 4. Lymphatic transport data are also included which describe the effect of co-administering a lipase inhibitor (tetrahydolipstatin, THL) with the formulations. The lymphatic transport of retinol and retinyl palmitate when administered as the micellar formulation was similar to the lymphatic transport of the retinyl ester when administered as the emulsified formulation. The tendency toward higher transport of retinyl palmitate when administered as the micellar formulation is consistent with the immediate availability of the vehicle lipids for absorption. There was a significant formulation effect, as the lymphatic transport of retinol administered as the micellar system was approximately 2.5 times greater than with the emulsion formulation. A previous study of cholesterol absorption by the same workers did not exhibit such a marked difference between emulsified or micellar formulations, although there was an indication of slightly greater transport from the micellar system.[200]

The presence of THL in the infusate significantly decreased the recovery of lipid in the lymph when administered as emulsified triglyceride, yet had minimal effect on lymph lipid transport when administered as the micellar formulation. These effects are consistent with the inhibitory action of THL towards lipase. The lymphatic transport of retinol was significantly reduced when THL was included in either the micellar or emulsified retinyl palmitate formulations, indicating the necessity for hydrolysis of the esterified retinol prior to absorption. It is likely that the enzyme responsible for the hydrolysis of cholesterol esters (pancreatic cholesterol ester hydrolase)[201] also hydrolyses the retinyl esters as well as the esters of the other lipid soluble vitamins. These data indicate that retinyl esters are hydrolysed prior to absorption and that retinol esters are the primary form of the vitamin present in lymph.

The micellar and emulsified formulation effects described by Fernandez and Borgström[199] are consistent with an earlier report which described a doubling of lymphatic transport when retinyl palmitate was administered as a micellar solution (containing polysorbate 80) rather than an emulsified formulation or oily solution. It was determined in a thoracic duct cannulated rat model that approximately 20% of the administered dose was lymphatically transported.[202] This study also examined the effect of fatty acid chain length and found that the rank order in terms of improved lymphatic transport was long chain > medium chain > short chain when administered as an oily solution. When administered as emulsion formulations, the effect of fatty acid chain length was no longer apparent.

It is clear that luminal factors play a major role in the lymphatic transport of the retinol esters, and interestingly, Nishigaki and co-workers[202] demonstrated that co-administration of long chain fatty acids was not an absolute requirement for lymphatic transport. These observations are consistent with a study by Noguchi and co-workers[203] who studied the lymphatic transport of retinyl acetate and found that, although the transport was low, there was little difference when administered as either a triolein emulsion or a polysorbate 80 micellar system which did not contain triglyceride. It was also demonstrated that the retinol present within the lymph after administration of either the lipid or micellar formulation was associated with a lipoprotein fraction of the lymph as determined by Sephadex gel chromatography. This suggests that the apparent appearance of lipoproteins may have involved endogenous lipids.

The lymphatic transport of vitamin E from different lipid-based preparations has been the subject of several studies because, in general, the transport of molecules with vitamin E activity is not considered to be as efficient as the other lipid soluble vitamins.[12] There are indications that the different tocopherols and tocotrienols are absorbed to different extents, although the general consensus appears to be that α-tocopherol is probably absorbed to the greatest extent.[12,204,205] Furthermore, the extent of absorption of tocopherol appears to be dependent upon age and maturity, although the basis for these effects are unknown.[206] Therefore, due to the different experimental protocols and study designs which have been utilized for studying tocopherol absorption, it is

unwise to attempt to over-interpret the various lymphatic transport data in the literature.

A unique property of α-tocopherol amongst the lipid soluble vitamins is an ability to interact (complex) in a noncovalent manner with fatty acids, and it has been suggested that this is the means by which α-tocopherol can stabilize cell membranes towards fatty acids.[207] The affinity of α-tocopherol for fatty acids was observed to increase with an increasing degree of unsaturation. It is not clear what effect this potential interaction may have upon the absorption and lymphatic transport of α-tocopherol when administered in different formulations.

The absorption of α-tocopherol has been recently studied in the human by following the appearance of administered d_3- or d_6-α-tocopherol in plasma lipoproteins.[208] Deuterated tocopherol was administered with a standard meal and the plasma lipoproteins were fractionated into the respective components. After administration, α-tocopherol first appeared in the chylomicron fraction of the plasma after which it was progressively apparent within the VLDL and other lipoprotein fractions. These observations are consistent with chylomicron-based transport from the intestine, uptake of chylomicron remnants by the liver, followed by secretion of newly formed VLDL with subsequent transfer to LDL and HDL.

The two major avenues which have been investigated for the promotion of tocopherol bioavailability are through formulation and prodrug-based approaches. The prodrug approach for the enhancement of tocopherol bioavailability is discussed in Chapter 5, and the following discussion is restricted to a summary of some recent formulation approaches.

Micellar solutions consisting of either bile salts or polysorbate 80 have been shown to enhance the lymphatic transport of α-tocopherol, and it was suggested that the enhancement was not related to the extent of solubilization by the micelle but was correlated with the size of the micelle and the net flux of water.[209] In these same studies it was demonstrated that the addition of lecithin to the micellar formulations decreased absorption. Other more recent studies have shown that lecithin (PC) dispersed preparations which contain medium chain triglycerides (MCTG) promoted the lymphatic transport of α-tocopherol relative to either a polysorbate 80 solubilized aqueous formulation or a soybean oil solution.[31,210] There was a doubling of lymphatic transport in thoracic duct fistulated rats when tocopheryl acetate was administered in a PC/MCTG formulation (23% of administered dose) relative to a PC-based formulation (11% of dose). Although the role of medium chain triglycerides and the promotion of the lymphatic transport of vitamin E has been previously described, the basis for the enhancement remains undetermined.[211,212] In these same studies, Fukui and co-workers[31] co-administered pluronic L-81, a reversible inhibitor of chylomicron formation, with the MCTG/PC/tocopheryl acetate formulation and found the lymphatic transport of tocopherol was markedly decreased, leading to the conclusion that the formation of chylomicrons

was essential for the lymphatic transport of tocopherol. This scenario implies that chylomicrons would have to be formed by the enterocyte to transport tocopherol in the absence of co-administered long chain fatty acids. A mass balance of the administered tocopherol would clarify whether (i) the MCTG actually enhanced the absorption of tocopherol from the intestine, (ii) whether the co-administered L-81 decreased the overall bioavailability of tocopherol leading to a decreased lymphatic transport, or (iii) if L-81 actually interfered with chylomicron formation in the absence of co-administered long chain fatty acids. Further studies are required to clarify these possibilities and to determine the relative effects of enhanced solubilization (bioavailability) and induced lymphatic transport. Aspects of the α-tocopherol lymphatic transport data are consistent with some of the vitamin A lymphatic transport data where absorption and lymphatic transport apparently occurred in the absence of co-administered long chain fatty acids.[202,203]

Long chain unsaturated fatty acids can decrease the absorption of the lipid soluble vitamins.[12] In studies where tocopherol was co-administered with unsaturated fatty acids, it was determined that the lower lymphatic transport was a function of decreased absorption (i.e., lower bioavailability), as there was a concomitant increase in the fecal excretion of tocopherol.[213] Hollander[194] studied the intestinal absorption of retinol in a perfused rat experiment and determined that decreased lymphatic transport when long chain fatty acids were added to the infusate solution was accompanied by an apparent increase in the portal blood absorption of retinol, and suggested that the unsaturated fatty acids altered the balance between lymphatic and portal transport of retinol. Although the mechanisms by which unsaturated fatty acids decrease the lymphatic transport of the lipid soluble vitamins remain undetermined, a number of different hypotheses have been advanced which include changes in the physical size of the mixed micelles or the partitioning between mucosal cells and the micellar phase,[12,194] alteration of relative absorption via the portal blood,[198] effects on active and passive transport pathways in the intestine,[214] and effects on lipoprotein formation.

The absorption of vitamin D from the intestine is complex and not fully defined, as there are significant contributions to the overall bioavailability from both portal blood absorption and lymphatic transport of the parent compound and active metabolites. Vitamin D_3 is transported in the lymph in the free form in association with intestinal lipoproteins, and the different hydroxylated metabolites of vitamin D_3, which are formed by the enterocyte, are primarily transported by the portal blood.[215–217] The lymphatic transport of vitamin D_3, the most nonpolar of the vitamin D sterols, is dependent upon solubilization in mixed micelles and subsequent formation of chylomicrons by the enterocyte. The absorption of the hydroxy- and dihydroxy-vitamin D_3 metabolites (which are also active) is not dependent upon bile salt solubilization or chylomicron formation, as these metabolites are predominantly absorbed by the portal blood. In a study of the relative portal blood or lymph transport of the vitamin

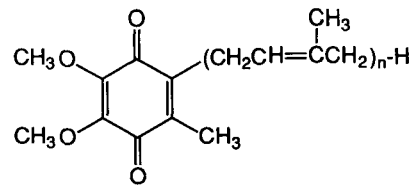


FIGURE 15. Structure of coenzyme Q_{10} where n = 10.

D sterols, Maislos and co-workers[215] concluded that the relative lymph/portal blood transport reflected the differential polarity of the compounds. It was determined that 1,25-dihydroxyvitamin D_3, which is the most polar metabolite, was absorbed directly into the portal blood and transport was not dependent upon lipid transport, whereas the less polar vitamin D_3 was transported via the lymphatics and was dependent upon lipid administration.[215] As expected, the differential absorption profile of the vitamin D sterols is manifested as a more rapid rate and earlier appearance of the dihydroxy metabolite transported by the portal blood as opposed to the slower and later appearance of the lymphatically-transported vitamin D_3.

2. Coenzyme-Q_{10}

Coenzyme-Q_{10} is a highly lipophilic benzoquinone derivative which plays a role in electron transport reactions and has utility in the treatment of congestive heart failure and angina pectoris. The structure of coenzyme-Q_{10} is depicted in Figure 15. The compound is currently administered intravenously as a soya-bean oil emulsion formulation in which the choice of emulsifying agent has a significant effect upon the tissue distribution and elimination of the compound from the plasma.[218,219] When administered orally, the majority of intestinal absorption is via the lymphatics. Katayama and Fujita[49] determined in a rat model that approximately 70% of the oral bioavailability of coenzyme-Q_{10} was related to intestinal lymphatic transport. However, in absolute terms only approximately 2% of the administered dose (0.6 mg/kg) was absorbed from the intestine. The lymphatic transport was approximately 1.5% of the administered dose when administered dissolved in HCO-60 (a commonly used polyethoxylated derivative of hydrogenated castor oil), and approximately 1% when administered as either a sesame oil or a 20 m*M* sodium taurocholate solution. It was demonstrated that the drug present within the lymph was primarily associated with the chylomicron fraction (80% of radioactivity), with the remainder associated with the VLDL fraction of the lymph. The percent of the administered dose transported by the lymphatics was independent of dose (range between 0.15 to 2.4 mg/kg) which indicated that the drug was probably absorbed from the intestinal lumen via a passive process.

3. Xenobiotics

Much attention has been focused on the role of intestinal lymphatic transport of lipid soluble xenobiotics as they are quite often toxic to animal

cellular systems and have the potential to accumulate in both body tissues and the food chain. The lymphatic transport of a range of different and specific lipid soluble xenobiotics has been previously discussed and should be consulted for further detail.[220] In terms of their lymphatic transport, there are numerous examples of the effects that different lipids have upon the extent of absorption and lymphatic transport. The major processes involved in the transport of lipophilic xenobiotics by the intestinal lymphatics are the same as have been discussed in terms of lipid digestion, solubilization, and triglyceride resynthesis, and the formation of chylomicrons by the enterocyte. These processes have been previously discussed in this chapter with reference to specific xenobiotics where appropriate data were available to substantiate the different factors discussed. As described in Section IV.B.2, an important point for consideration in terms of lymphatic transport is the likely metabolism of the xenobiotic which can occur within the enterocyte during lipid/xenobiotic absorption, and the consequent effects on lymph transport of the compounds and portal blood transfer of the metabolites. A further factor is the potential effects of absorbed xenobiotics on the numerous metabolic functions of the enterocyte. The major functions of the enterocyte which can be affected by the absorbed lipophilic xenobiotics are those involved in lipid digestion and transport as the lipid digestion products provide the vehicle for access to the different systems within the cell. Bell[221] has comprehensively reviewed the effects of a large number of lipid soluble xenobiotics on lipid metabolism and described the different effects on either triglyceride resynthesis, phospholipid synthesis, cholesterol ester formation, and effects on apoprotein synthesis. The effects on the assembly and secretion of lipoproteins were also described.

4. Cyclosporin

The effective oral delivery of cyclosporin, a cyclic undecapeptide, is a major clinical goal, as it is a widely used immunosuppressant drug in liver, heart, kidney, and bone marrow transplantation. The absolute bioavailability of cyclosporin in human patients, although highly variable, has been estimated at approximately 20 to 30%,[222] which is in good agreement with data reported in the rat.[9] The various factors which effect the systemic bioavailability of cyclosporin have been recently reviewed.[223,224]

Cyclosporin is considered to be highly lipophilic as the log P is approximately 3, the solubility in lipid-based solvents is reasonably high, and the aqueous solubility is less than 10 μg/ml.[225] It is believed that the immunosuppressive activity of cyclosporin is related to a selective action on lymphocytes which play a key role in the induction of immune responsiveness. Therefore, as lymphocytes circulate throughout the lymph and the lymphatic system, it has been postulated that the concentration of cyclosporin in the lymph is most likely a determinate of therapeutic efficacy which could be enhanced by improved delivery of drug to the lymphatic system. In the absence of selective lymphatic transport, the presence of cyclosporin in the lymph is due to transfer

TABLE 5
Cumulative Excretion of Radioactivity in Urine and Bile After Administration of [^{3}H]Cyclosporin in Either Olive Oil or Medium Chain Triglyceride (MCTG) Vehicles to Bile Duct Cannulated Rats

Vehicle	Cumulative excretion[a] (% dose administered)
Olive oil (nondigested)	32.1 ± 0.6
MCTG (nondigested)	23.6 ± 1.7
Olive oil (pre-digested)[b]	47.9 ± 4.7
MCTG (pre-digested)	39.0 ± 5.4

[a] Data are reported as Mean ± SE.
[b] Pre-digestion refers to *ex vivo* digestion prior to administration.

Taken from Reymond, J.-P., Sucker, H., and Vonderscher, J., *Pharm. Res.*, 5, 677, 1988. With permission.

from the blood after either intravenous administration or absorption via the portal blood following oral dosing.[226]

The intestinal lymphatic transport of cyclosporin in rats after administration of an olive oil/cyclosporin solution was either 0.35% (6.5 mg/kg dose) or 0.47% (25.2 mg/kg) of the orally administered dose.[9] It was determined in these studies that the fraction of the administered cyclosporin dose which was absorbed from the olive oil solution was 21%. When the lymphatic transport data were corrected for bioavailability effects, the transport represented approximately 2% of the absorbed dose. The conclusion from these studies was that the limiting factor in the systemic bioavailability of cyclosporin was not related to a high first pass effect but rather poor absorption from the dose form. This is in agreement with human data which have demonstrated slow and incomplete absorption of cyclosporin.[222]

The approaches which have been taken to enhance the bioavailability and lymphatic transport of orally administered cyclosporin fall into three categories: (i) choice of co-administered lipid, (ii) application of lipid-surfactant mixed micelles systems, and (iii) solubilization of cyclosporin with potential absorption promotors.

The bioavailability of cyclosporin administered in either a medium or long chain triglyceride vehicle evaluated in a rat model indicated that, although lymphatic transport was expected to contribute minimally to the overall bioavailability, there were still significant effects of the lipid on the absorption of the administered dose.[227,228] Table 5 lists the cumulative excretion of radioactivity following the administration to rats of [^{3}H]cyclosporin dissolved in either olive oil or a medium chain triglyceride (MCTG). There was a significant increase in radioactivity appearing in the urine and bile when administered as the olive oil rather than the MCTG formulation. When the vehicles were pre-

digested *ex vivo*, there were trends toward greater transport from the olive oil formulation, although it did not reach statistical significance. In both cases, the *ex vivo* pre-digestion of the administered lipid increased the effective bioavailability relative to the undigested lipid. As lymphatic transport of cyclosporin is not expected to be a major contributor to bioavailability with these vehicles,[9,226] the differences in transport are most likely related to events that are altering the portal blood transport of the compound. It was suggested that the differences between the vehicles were most likely related to different effects upon the permeability of the intestinal mucosa.[227] The *ex vivo* pre-digestion of lipids has also been observed to enhance the bioavailability of other poorly water soluble drugs.[229,230]

Some interesting data have been reported describing the enhancement of the lymphatic delivery of cyclosporin using mixed micellar systems with a range of different solubilizers.[231–233] A major finding of these studies was the increased lymphatic delivery of cyclosporin in the apparent absence of enhanced chylomicron-based transport. The increased transport of cyclosporin to the lymphatics was observed in terms of both increased drug mass and concentration with typical increases in the range of 20- to 50-fold relative to standard olive oil formulations. Table 6 lists the lymphatic transport of cyclosporin as a function of different formulations administered to thoracic duct cannulated rats and Figure 16 depicts the time-dependent lymph concentrations of cyclosporin. The effect of HCO-60 and sugar ester solutions on the lymphatic transport were marked and represented 10- to 20-fold increases over the cumulative lymphatic transport achieved with either of the two lipid-based formulations. Although the mixed micelle formulation enhanced the transport relative to the lipid-based systems, the cumulative transport was approximately 50% of that observed with the solubilized systems. The systemic plasma levels of cyclosporin exhibited a similar rank order as was observed with the lymphatic transport data, although the magnitude of the differences between the formulations were marginal. This is consistent with the plasma levels of cyclosporin, reflecting absorption through the portal blood in these thoracic duct cannulated rats, and therefore, the formulation effects are apparently more specific for the lymphatic rather than the portal blood route of absorption/transport. The effect of the solubilized HCO-60 formulation was evaluated relative to a sesame oil formulation in a mouse skin-allograft survival rate model. The percent survival rate (efficacy) in a group receiving a 1 mg/kg HCO-60 formulation was midway between the survival rates observed for the groups receiving either a 7 or 15 mg/kg sesame oil formulations of cyclosporin. These efficacy data parallel the observed effects of the formulation on the lymphatic transport of cyclosporin. These results have been confirmed in a rat heart transplant model where the survival time was significantly longer with the solubilized formulation rather than an olive oil formulation.[234]

The mechanisms by which the solubilized systems increased the appearance of cyclosporin in the lymph remain undetermined. From the experiments reported by Takada and co-workers,[231–234] it does not appear that the increased

TABLE 6
Lymphatic Transport of Cyclosporin in Thoracic Duct Cannulated Rats as a Function of Different Orally Administered Formulations

Formulation[a]	Cumulative Transport[b] (% dose over 0- to 6-hr period)
Sesame oil	0.19 ± 0.04
Linoleic acid	0.05 ± 0.62
Mixed micelle solution	0.66 ± 0.19
HCO-60 solution	2.14 ± 0.04
Sugar ester solution	1.62 ± 0.92

Note: The dose of cyclosporin was constant (7 mg/kg) in the different formulations.

[a] The sesame oil and linoleic acid formulations were prepared by dissolving cyclosporin in the appropriate lipid. The HCO-60 (polyethoxylated hydrogenated castor oil) formulation was prepared as an 8% w/v solution; the sugar ester (DK F-140) formulation was prepared as a 0.2% w/v. The mixed micellar formulation consisted of 5% w/v linoleic acid and 8% w/v HCO-60 and was prepared by sonication.

[b] Data are presented as Mean ± SE.

Taken from Takada, K., Yoshimura, H., Yoshikawa, H., Muranishi, S., Yasumura, T., and Oka, S., *Pharm. Res.*, 3, 48, 1986. With permission.

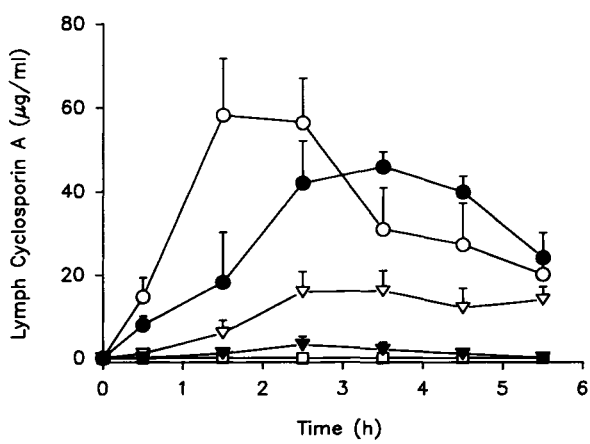


FIGURE 16. Concentrations of cyclosporin A appearing in thoracic duct lymph of rats after oral administration of cyclosporin A (7 mg/kg) prepared with different solubilized formulations. HCO-60 solution (○); sugar ester (DK ester F-140, ●); mixed micellar system (△); sesame oil (▼), and linoleic acid (□). The volume administered was 1 ml/kg body weight and the data are presented as mean ± SE. (From Takada, K., Yoshimiura, H., Yoshikawa, H., Muranishi, S., Yasumura, T., and Oka, T., *Pharm. Res.*, 3, 48, 1986. With permission.)

lymphatic transport is a function of enhanced chylomicron transport as there was no difference in the lymphatic transport of cyclosporin if either milk (which would stimulate chylomicron formation) or saline was pre-administered to the rats.[232] It appears that the effect of the solubilizers may be related to the general absorption-enhancing properties of these materials. Further studies in the rat have demonstrated that bile is necessary for the enhanced delivery of cyclosporin from HCO-60 solubilized formulations and that other formulations such as propylene glycol and polyethylene glycol can also affect the relative systemic or lymphatic availability of cyclosporin.[233] The effect of emulsion particle size,[235] formulation of lecithin-based lipid microspheres,[236] and solid enteric dispersions[237] have also been shown to enhance the absorption and lymphatic transport of cyclosporin. The mechanisms of the promotion of the lymphatic availability of cyclosporin appear to occur through pathways which are not primarily dependent upon chylomicron formation and require further investigation.

5. Other Approaches

One area where lymphatic transport is being actively pursued is in the design of lipophilic prodrugs. Chapter 5 describes the evaluation of a range of lipophilic prodrugs designed specifically for lymphatic transport and the successes and failures of this approach. An additional area in which lymphatic transport has been investigated is in the transport of orally administered polar compounds which is addressed in Chapter 7. This form of lymphatic transport is not dependent upon lipoprotein formation and consequently will not be addressed in this chapter.

E. PREDICTION OF INTESTINAL LYMPHATIC TRANSPORT

A major goal in the pharmaceutical sciences is an understanding of the relationship between the physicochemical properties of an administered drug and the likely extent of absorption from the gastrointestinal tract.[238] Although the majority of research in this area has focused upon the absorption of drugs via the portal blood, the physicochemical and biological factors which govern intestinal lymphatic drug transport lend themselves to such an examination because the chylomicron primarily provides the basis for drug transport when co-administered with long chain fatty acid vehicles.[3,4,8] The following description of the potential estimation of intestinal lymphatic transport assumes that the transport mechanism is primarily lipoprotein-based and that absorption promotors or other extrinsic factors are not the basis for the observed lymphatic transport.

Extensive lipophilicity is the most commonly described physicochemical property considered necessary for lymphatic transport and is often referred to in terms of a high partition coefficient which represents an equilibrium partitioning of the drug between an organic phase (commonly, 1-octanol) and an aqueous phase. An apparent relationship between lipophilicity estimated by

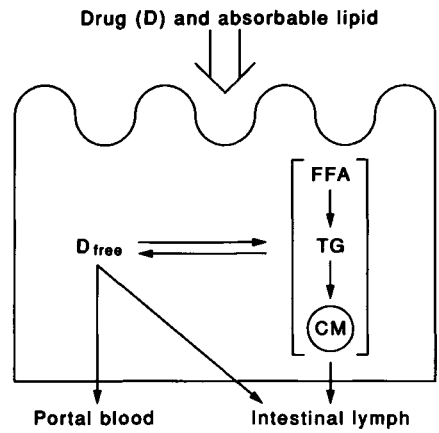


FIGURE 17. Schematic representation demonstrating the importance of partition coefficient in determining intestinal lymphatic transport. Absorbed fatty acids (FA) and monoglycerides are resynthesized into triglyceride (TG) which is incorporated into chylomicrons (CM). (From Stella, V. J. and Charman, W. N., *Novel Drug Delivery*, Wiley and Sons, 1989, chap. 6. With permission.)

log P and the extent of lymphatic transport has been demonstrated for, among others, a series of DDT analogs[14] and testosterone esters.[158] A simple description of the role that a high partition coefficient plays in the lymphatic transport process is depicted in Figure 17. This simple illustration depicts the relative partitioning of an administered drug between the aqueous and lipid phases present within the cell and intestinal lumen, and ignores the potential role of luminal degradation, intracellular drug metabolism, and either intracellular or extracellular binding of the drug as they may decrease the extent of lymphatic transport. Furthermore, it tacitly assumes the optimal formation of chylomicrons in response to the administered lipid.

The flow rate ratio of portal blood to intestinal lymph is approximately 500:1.[239,240] As described in Section III B, the transport of lipophilic compounds via the intestinal lymphatics is primarily in association with the chylomicron fraction of the intestinal lipoproteins produced by the enterocyte in response to ingested lipid. Therefore, it is the chylomicron flux that is the factor which determines lymphatic transport and not the actual volumetric flow of intestinal lymph.[8,15] Although chylomicron formation is phasic and dependent upon the administration of appropriate lipid, as a general guide, the lipid content of intestinal lymph during periods of fat transport is approximately 1 to 2%.[14,42,119] Therefore, for an absorbed compound to be equally transported by both the portal blood and the lipid fraction of the intestinal lymph, the effective partition coefficient of the administered drug would need to be approximately 50,000:1. This value translates to a log P (partition coefficient) between the portal blood and the lipid content of the intestinal lymph of 4.7.

Although this analysis is consistent with log P values associated with significant lymphatic transport, it is based upon equilibrium measurements and therefore ignores the dynamic nature of drug absorption and the resynthesis of lipoproteins by the enterocyte.

It is inappropriate to rely solely upon a large partition coefficient as a predictor of lymphatic transport, as the partitioning of a lipophilic drug between an aqueous phase and an organic solvent such as 1-octanol will not necessarily translate to a high solubility in a triglyceride lipid. Furthermore, a high solubility in an organic solvent such as 1-octanol or chloroform will not always relate to a high solubility in a poorly solvating, largely hydrocarbon solvent such as a triglyceride. This treatment assumes that the solubility of the drug in the lipid interior of the chylomicron is not a limiting factor in transport and that there are no specific interactions of the drug with components of either the lymph or the blood.

Preferential drug binding to cellular fractions or components of the blood or lymph may alter the effective partition coefficient of the drug (e.g., drug binding to red blood cells). It has been recently demonstrated that the lower than expected lymphatic transport for a compound (penclomedine),with a high lipid solubility (175 mg/ml in peanut oil) and a high partition coefficient (log P (octanol/water) of 5.5) was related to high binding for red blood cells (RBC:plasma ratio of 8:1), which effectively decreased the magnitude of the partition coefficient which resulted in decreased lymphatic transport.[241]

Charman and Stella[17] have indicated that it is possible to predict the potential maximal extent of chylomicron-based transport of lipophilic compounds simply from analysis of the lipid (triglyceride) solubility of the compound and the flux of chylomicron lipid transported in the lymph. Caveats with this analysis include incomplete drug absorption and the potential for drug metabolism. Assuming that a lipophilic drug (log P > 5) is maximally present at approximately 25% of saturated solubility in the triglyceride lipid of the lymph,[8,50] then the maximal transport of drug will be the product of the lipid solubility and the mass of triglyceride transported via the lymphatics. The utility of this calculation, which will almost certainly overestimate the extent of transport, is that it gives an estimate of the maximal lymphatic transport and hence an indication of the potential role of lymphatic transport in the observed bioavailability of the lipophilic drug.

V. PERSPECTIVES

Although intestinal lymphatic transport is a highly complex process, it offers many potential pharmaceutical advantages for orally administered drugs. These include a potential avoidance of the first pass effects, delivery to various regions within the lymphatic system, and the possibility of controlling the rate of entry of compounds into the systemic circulation. For small(er) molecular weight lipophilic compounds, it is clear that those transported by the intestinal

lymphatics are associated with, and dependent upon, the formation of lipoproteins (primarily chylomicrons) by the enterocyte. For peptide, and potentially protein-based drugs, the promotion of lymphatic transport is an area of growing interest. Advances in this area of drug delivery will be made through an appreciation of the sequential processing characteristics of lymphatic transport and the different means for promoting access to the intestinal lymphatics.

At this time, the processes of lipid digestion, absorption, and the resynthesis of the absorbed lipid digestion products into lipoproteins by the enterocyte are yet to be fully understood, as is the "association" of lipophilic drugs with co-administered lipid during these processes. Notwithstanding the gaps in our current knowledge, formulation practices for promoting the lymphatic transport of lipophilic molecules are evolving through an appreciation of the biochemical steps involved in the transport of ingested lipid and the relevance of the physicochemical properties of the administered drug. As these processes and the intracellular processing of the drug and lipid are better understood and characterized, the potential of intestinal lymphatic drug transport as a contributor to drug bioavailability can be realistically assessed.

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Chapter 5

LIPOPHILIC PRODRUGS AND THE PROMOTION OF INTESTINAL LYMPHATIC DRUG TRANSPORT

Valentino J. Stella and Nancy L. Pochopin

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I. INTRODUCTION

A. DEFINITION OF PRODRUGS

Prodrugs are molecules that must undergo biotransformation prior to exhibiting their therapeutic effects.[1–3] First defined by Albert,[4] but practiced earlier, prodrug approaches to modified drug delivery have principally been used to overcome various shortcomings of therapeutic agents having some barrier to their optimal usefulness. The prodrug concept is depicted in Figure 1 where the drug is chemically modified by the addition of a "promoiety" that alters the chemical, physical, and/or biochemical properties of the drug allowing it to overcome the barrier to its usefulness. The bond between the drug and promoiety is usually of the covalent type, although Stella[3] has argued that complexes and some salts might also be considered as prodrugs. The reversion of the prodrug to drug is by enzymic or chemical reaction, but, if complexes and salts are considered as prodrugs, simple dissociation by dilution may also be involved.

Prodrugs have traditionally been used to overcome barriers such as poor permeation across biological membranes, including those of the gastrointestinal tract and the blood brain barrier, and poor aqueous solubility that may limit dissolution in the gastrointestinal tract and/or the ability to administer the drug via the parenteral route in a physiologically acceptable vehicle. Examples of these and other barriers that have been overcome through prodrug manipulations have been extensively reviewed.[1–3]

Prodrug studies to effect delivery to the lymph have been limited. Also, in a number of the studies, it is not clear whether the chemically modified agent is behaving as a prodrug or as an analog. In some studies, improvements in drug delivery have been attributed to improved delivery to the lymph without real evidence, while in others, alterations in drug delivery profiles may have inadvertently resulted from lymph delivery.

The major focus of this chapter, therefore, will be to critically review the available data pertaining to the manipulation of small molecule delivery to the lymph. The emphasis will be on delivery to the intestinal lymphatics after oral drug/prodrug administration because of the greater interest in oral drug delivery compared to parenteral routes of administration. The various vehicle and formulation aspects of intestinal lymphatic transport were described in the preceeding chapter (Chapter 4). Chapters 7 and 8 address the issues of drug delivery from macromolecular prodrugs (small drug with a macromolecular carrier) after parenteral delivery and consider some examples of polar drug transport (as ionic complexes) via lymph from the lower intestinal tract. An effort will be made to relate these findings to the overall question of whether prodrugs can be used to effectively promote the delivery of drugs to the lymph.

The delivery of agents to the lymphatics is not only dependent on the physical/chemical and metabolic stability properties of the drug/prodrug, but also on the route of administration and the vehicle in which the drug is administered. Additionally, the methodology used in the assessment, including

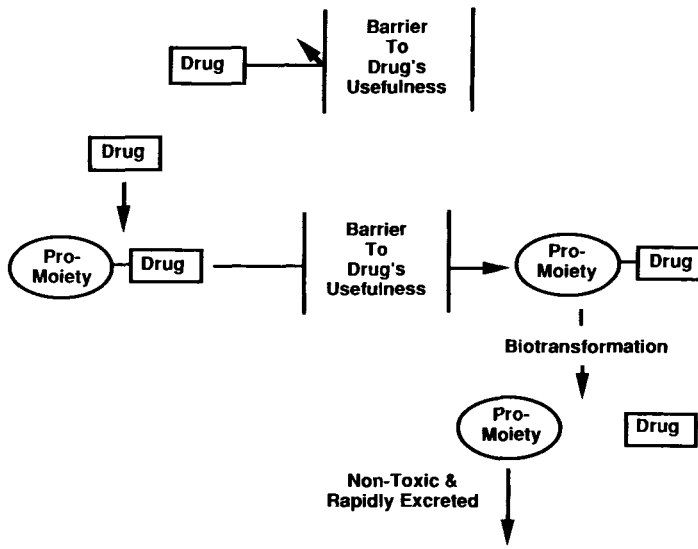


FIGURE 1. Schematic representation of the pertinent properties of a prodrug.

the experimental animal model used, can positively or negatively influence the conclusions (Chapter 3), making comparisons across studies difficult.

B. WHY ATTEMPT TO PROMOTE LYMPHATIC DELIVERY VIA PRODRUGS?

The major reasons for wanting to promote lymph delivery of drugs via prodrugs are the same as those listed for attempting to manipulate drug delivery via vehicle and route of administration changes (formulation variables), namely:

1. Avoidance of presystemic metabolism after oral dosing
2. Targeting of drugs to the lymph
3. Modulated input rate to the body

The prognosis for being able to manipulate drug transport to the lymph via formulation changes is poor when the physicochemical properties of the drug are limiting. Therefore, if the properties of the drug can be modified in a bioreversible manner, e.g., by conversion to a prodrug with the "right" properties for lymph transport, delivery via this route should be possible, thus the strength of the prodrug approach to altered drug delivery.

C. WHY HAVE MOST ATTEMPTS AT PROMOTING LYMPH DELIVERY AFTER ORAL DOSING VIA PRODRUGS MET WITH ONLY MARGINAL SUCCESS?

The complexity of lymphatic drug delivery after oral dosing is poorly appreciated, and therefore, overly simplistic hypotheses have been employed

in attempts to promote lymphatic delivery. Consider the following working hypothesis:

"Since most drugs that are transported in the lymph after oral dosing are very lipophilic and my drug, while undergoing a significant degree of presystemic clearance, is relatively hydrophilic, I will synthesize a very lipophilic ester of my drug which has a high octanol/water partition coefficient. After oral dosing and absorption, followed by chylomicron associated intestinal lymph transport, this prodrug will break down and release my drug, thereby circumventing its presystemic metabolism."

What are the possible flaws in this hypothesis?

1. The hypothesis appears to place an overemphasis on the importance of partition coefficient, as measured by the octanol/water partition coefficient and usually defined as log P, as the critical determinant in controlling lymph transport. While high log P values are essential for chylomicron associated lymph transport[5,6] because they give an indication of the likely affinity of the molecule for chylomicrons, the magnitude of the "required" partition coefficient is not always appreciated. While a drug with a log P value of 2 to 3 might be considered lipophilic by most standards, log P values of > 6 appear to be necessary for lymph transport. The log P value should be determined at the physiological pH value of 7.4 where many drugs/prodrugs may be ionized. For example, an acidic drug may have a high log P value at acidic pH values but a relatively low log P at pH 7.4. Similarly, a basic drug may have a large log P in its free base form at high pH values, but it will be protonated at pH 7.4, thereby lowering the effective log P value of the drug/prodrug. Additionally, an octanol/water partition coefficient may not be reflective of the affinity of the drug/prodrug for chylomicrons and their remnants. Principle components of chylomicrons are various long chain triglycerides whose bulk and microscopic properties are different from octanol. The chylomicron should not even be treated as a droplet of oil because other components, such as cholesterol and its esters, phospholipids and surface-bound proteins make specific interactions possible between the drug/prodrug and the surface of the chylomicron. These interactions will not be reflected by the relative affinity of an agent for a bulk solvent like octanol.

2. The implication that lipophilicity is best defined by a partitioning phenomena does not take into account the fact that lymph transport may become capacity limited i.e., drugs can have large log P values and high affinities for organic phases such as octanol (relative to water), yet the drug may be poorly soluble in either of the different phases.[7] The transport of a lipophilic compound in lymph in association with chylomicrons requires not only that the drug/prodrug have an affinity for the chylomicron relative to the aqueous fraction of lymph, but it must have the capacity to "dissolve" in that phase.[7]

3. The hypothesis described above assumes that an ester prodrug (providing it is able to dissolve in the contents of the gastrointestinal tract and be

absorbed, see the following point), can be designed to be stable within the contents of the gastrointestinal tract and the intestinal cells but then be cleaved in the plasma or some peripheral tissue. Esterases and some peptidases that are capable of cleaving ester prodrugs are present in most organs of the body but are particularly active in the intestinal tract, intestinal cells, and the liver. Therefore, there is a low probability that an ester prodrug would dissolve in the gastrointestinal tract, survive the luminal and brush border esterases, be absorbed and survive the esterases in the enterocytes, strongly associate with prechylomicrons and chylomicrons and then be transported into the mesenteric lymphatics. Assuming that the ester was capable of surviving the hostile environment of the absorption process, in all probability it would convert to the parent drug in the liver — the organ likely responsible for the presystemic metabolism of the parent drug in the first place. Therefore, the likelihood of a sequential first pass effect is high[8] due to the liberation of the drug in the liver and the possibility of "sequential metabolism".

4. A possible fatal flaw in the proposed hypothesis is that in making the prodrug more lipophilic, and therefore less water soluble, dissolution of the drug in the gastrointestinal tract would be slow and could limit the absorption process. Although the relationship between aqueous solubility and *in vitro* dissolution of drugs is well understood,[9] the *in vivo* behavior of poorly water soluble drugs is not well predicted by aqueous dissolution studies.[10] Nevertheless, adequate aqueous solubility is essential for absorption as the lack of sufficient solubility will ultimately be detrimental to bioavailability or lymph transport (or both). Figure 2 illustrates the concept that for a given prodrug series (and assuming all other factors to be nonrate limiting) the relationship between a potentially increased extent of lymph transport and lipophilicity is likely to be parabolic with maximal transport occurring within a relatively narrow region of the lipophilic/hydrophilic balance band. The maximum extent of transport in this case would likely be considerably less than 100%, with factors such as choice of vehicle and metabolic stability affecting both extent of transport and the broadness of the band.

D. WHAT DOES THE FUTURE HOLD?

From this qualitative discussion and the literature described in the previous chapters, it is obvious that lymph transport is a highly complex process. Therefore, the ability to prospectively manipulate lymph transport via prodrug modifications will have to be "smarter" than most efforts have been in the past. The simple process of preparing a lipophilic prodrug through a simple esterification process is not likely to lead to success. On the other hand, a better understanding of the complexity of the lymph transport process, and research leading to the design of promoiety linkages that are selectively cleaved may lead to chemical manipulations that are incapable of undergoing bioreversion during absorption but undergo conversion at some post-absorption site other than the liver. Such a prodrug approach may prove very successful. The

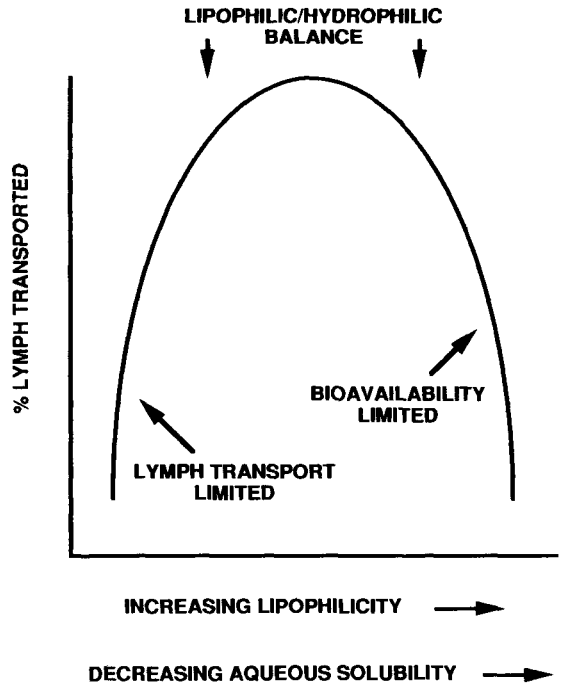


FIGURE 2. Relationship between the extent of intestinal lymphatic transport of a drug and lipophilicity after oral administration.

prophetic words of Adrian Albert should be kept in mind: "... although a detailed knowledge of permeability and enzymes can assist a designer in finding pro-agents ... he will have in mind an organism's normal reaction to a foreign substance is to burn it up for food." [11]

Despite the slightly pessimistic view just expressed, it is valuable to review examples of attempts to use prodrugs to promote lymph transport. A review of both the successes and failures in this area can provide valuable insight into the directions for future prodrug research in this field.

II. LIPOPHILIC PRODRUGS DESIGNED TO AVOID PRESYSTEMIC METABOLISM BY PROMOTING LYMPHATIC TRANSPORT

Although the avoidance of presystemic clearance is often stated as a primary justification for promoting intestinal lymph transport, few positive examples exist in the literature. However, from an anatomical standpoint the concept is sound and Figure 3 depicts the pertinent anatomical features of the circulatory systems and the relative transfer processes involved. As illustrated in Figure 3, a drug that is absorbed from the lumen of the gastrointestinal tract may undergo presystemic metabolism in either the lumen, brush border of the

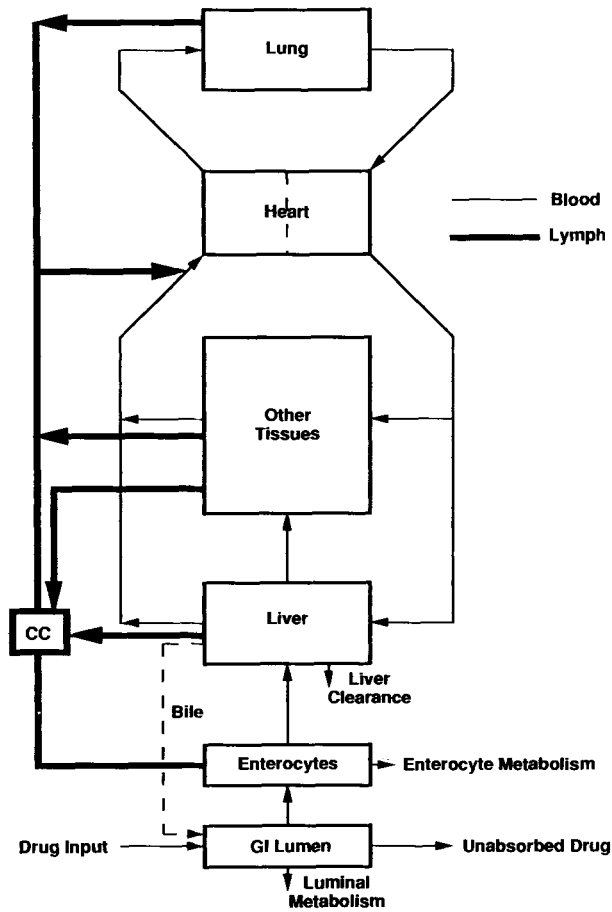
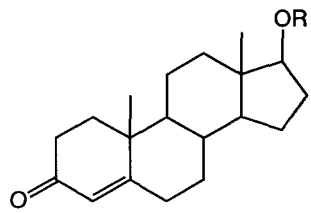


FIGURE 3. Physiological pharmacokinetic model showing the relationship of venous and arterial blood flow to lymph flow after oral drug input.

intestinal cells, within the enterocytes, or in the liver prior to reaching the systemic circulation. On the other hand, if the drug is given as a prodrug which can survive the luminal and intestinal metabolic assault and be transported via the intestinal lymph, then it could reach the systemic circulation while bypassing the liver. Providing the prodrug is cleaved to drug at a site other than the liver,[8] then intestinal lymphatic drug transport could circumvent the hepatic presystemic clearance of the drug.

A. SYSTEMIC DELIVERY OF STEROIDS

Testosterone is used to treat androgen deficiency syndromes in men. Although depot parenteral forms of testosterone esters can provide prolonged therapy, oral testosterone therapy would be more convenient and therefore



Testosterone: R=H

Testosterone undecanoate: R= $-COC_{10}H_{21}$

FIGURE 4. Structure of testosterone and its undecanoate ester.

more desirable. Testosterone undergoes nearly complete liver presystemic clearance after oral dosing,[12,13] and unless very high doses are administered, oral testosterone therapy is inadequate.[14] Testosterone undecanoate was commercialized as an orally available form of testosterone (Figure 4), although Kishimoto[15] has reported that testosterone esters may have intrinsic pharmacological activity of their own. Sieber et al.[16] have shown that testosterone is not lymphatically transported while Coert et al.[17] demonstrated the exclusive absorption of testosterone undecanoate by the intestinal lymphatics.

Noguchi et al.[6] determined the lymphatic transport in a conscious rat model of a series of testosterone esters (heptanoate, undecanoate, palmitate, and oleate) and attempted to correlate the degree of lymph transport of the esters and free testosterone to the logarithm of the heptane/water partition coefficients. The esters were administered in either oleic acid or other lipophilic solvents. A reasonable linear correlation, depicted in Figure 5, was observed between the logarithm of the extent of testosterone transport (ester plus free testosterone, percent of administered dose) and the logarithm of the heptane water partition coefficient. A similar correlation between the transport of free testosterone and the respective partition coefficients did not exist.

Although a larger amount of the testosterone palmitate ester was transported lymphatically, the undecanoate ester produced the highest amount of free testosterone in the lymph. No testosterone was observed after the palmitate ester. Testosterone undecanoate has a log P between *n*-heptane/water of about 6.5, making it as lipophilic as DDT and other drugs that are lymphatically transported. Therefore, the relatively poor performance of testosterone undecanoate as a lymphatically-transported molecule can probably be traced to its presystemic metabolism to testosterone and/or other metabolites.

The cumulative percentage of testosterone undecanoate transported intact in the lymph was only 0.5% of the administered dose and when a bioavailability study was performed, Noguchi et al.[6] determined that the oral bioavailability of testosterone undecanoate was 0.39% compared to a control I.V. dose. These

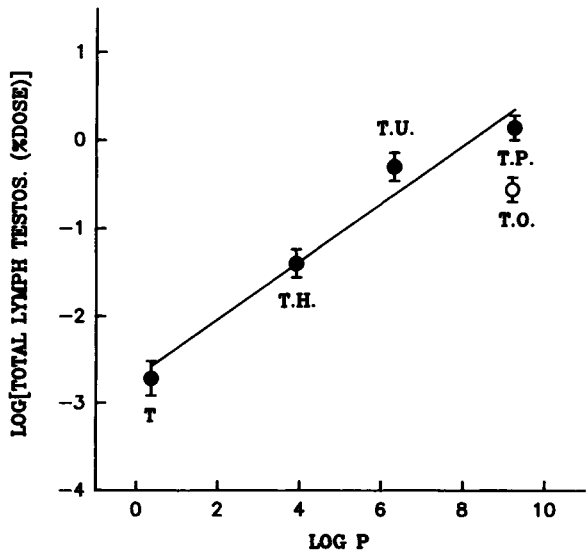


FIGURE 5. Relationship between the logarithm of total testosterone equivalents appearing in 24 hr mesenteric lymph (% administered dose, Mean ± S.E.) and the logarithm of their respective n-heptane/water partition coefficients for a series of testosterone (T) esters. T.P., T. palmitate; T.O., T oleate; T.U., T. undecanoate; T.H., T. heptanoate; and T, testosterone. (From Noguchi. T., Charman, W. N., and Stella, V. J., *Int. J. Pharmaceut.*, 24, 173, 1985. With permission).

data suggest that only that fraction of testosterone undecanoate transported by the intestinal lymphatics reached systemic circulation intact, i.e., as a percentage of the absorbed dose the lymphatic transport of intact testosterone undecanoate was close to quantitative. Horst et al.[18] have shown that 5-α reduction of testosterone undecanoate occurs in the lumen of the intestine and that this reduced form of testosterone undecanoate is transported in intestinal lymph. The analytical procedure used by Noguchi et al. did not assay for the 5-α reduced material.

Recently Tauber et al.[19] studied the bioavailability of testosterone after oral administration of either testosterone undecanoate or free testosterone in young women. They confirmed the high metabolic clearance of testosterone and estimated the systemic availability of testosterone to be 3.56 ± 2.45%. After administration of the undecanoate ester, the absolute bioavailability of testosterone increased to 6.83 ± 3.32%. Although these results are not dramatic, they are consistent with the undecanoate bypassing some of the presystemic metabolism of testosterone after oral dosing, and based on the available animal data, this is most likely the result of lymph transport.

Lipoidal esters, ethers, and enol ethers of various steroids have shown encouraging activity after oral dosing from lipid vehicles and after fatty

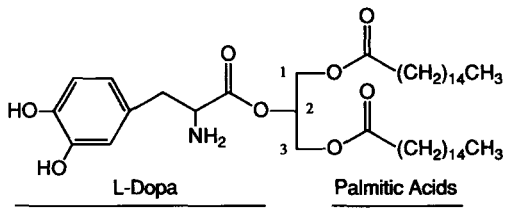


FIGURE 6. Chemical structure of a glyceride prodrug of L-Dopa: 1,3-dihexadecanoyl-2-[(S)-2amino-3-(3,4-dihydroxyphenyl)propanoyl]propane-1,2,3-triol.

meals.[20–24] In some cases, a significant prolongation of the activity was also noted. Although lymphatic transport was not assessed in these studies, it may be speculated that lymph transport played a role in increasing the systemic availability of these steroid derivatives. The results may suggest that bioreversible ethers rather than esters may prove more useful in enhancing the oral availability of presystemically cleared steroidal drugs by promoting lymph delivery.

B. CONJUGATED DRUGS

A strategy to circumvent the presystemic metabolism of phenolic drugs would be most welcomed as the bioavailability of this class of drugs is often severely compromised. Attempts at designing prodrugs to block the conjugation of phenols have met with only limited success. Rather than blocking the hydroxyl groups of L-Dopa in an attempt to prevent conjugation, Garzon-Aburbeh et al.[25] evaluated a different strategy by preparing a glyceride derivative of L-Dopa (Figure 6). L-Dopa undergoes significant presystemic metabolism by conjugation as well as metabolism by other routes, including decarboxylation to dopamine. Systemic conversion to dopamine accounts for many of the side effects associated with L-Dopa therapy for the treatment of Parkinson's disease.

The hypothesis of Garzon-Aburbeh et al.[25] for designing the glyceride prodrug of L-Dopa was that it should behave as a triglyceride mimic and would, therefore, be lymph promoting or lymphotropic. A comparative study of L-Dopa and dopamine levels in lymph and plasma after oral L-Dopa vs. prodrug administration conducted in mice, rats, and monkeys revealed some interesting findings.

The cumulative amount of L-Dopa found in lymph during the 5-hr post-dosing period after L-Dopa administration was 0.19% of the administered dose. However, after prodrug administration, 23.2% of the dose appeared in lymph, with $8.3 \pm 0.5\%$ as intact prodrug and $14.9 \pm 0.9\%$ as related glycerides. The authors defined related glycerides as spectroscopically and chromatographically similar materials to the prodrug and they assumed that these species were glycerides where the 1- and/or 3- palmitic acid (hexadecanoic acid) residues of the prodrug had been replaced by other endogenous fatty acids

TABLE 1
Comparison of Some Pharmacokinetic Parameters in Three Animal Species After Oral Administration of Equimolar Doses of L-Dopa and a Glyceride Prodrug of L-Dopa

Animal species	Compd. admin.	L-Dopa			Dopamine		
		Cp_{max}[a]	t_{max}[b]	AUC[c]	Cp_{max}[a]	t_{max}[b]	AUC[c]
Mouse	L-Dopa	1.12 ± 0.32	30	ND[d]	4.73 ± 0.49	30	ND
	Prodrug	1.60 ± 0.21	120	ND	3.54 ± 0.19	120	ND
Rat	L-Dopa	16.2 ± 2.6	60	61.4	52.0 ± 1.7	180	236.7
	Prodrug	24.6 ± 3.2	180	119.9	17.1 ± 2.2	240	81.9
Monkey[e]	L-Dopa	9.5	25	ND	8.1	90	ND
	Prodrug	18.3	110	ND	6.1	150	ND

[a] Maximum observed plasma concentrations (nmol/ml).
[b] Time (min) when maximum plasma levels were observed.
[c] Values for the Area Under the plasma concentration versus time Curves (AUC, nmol.hr/ml) were estimated from the data in figure 4 of reference 25.
[d] Not determined.
[e] These data were determined in a 2.7 kg Cynomolgus monkey fasted for 12 hr (n = 1).

Taken from Garzon-Aburbeh, A., Poupaert, J. H., Clasen, M., and Dumont, P., *J. Med. Chem.*, 29, 687, 1986. With permission.

according to the normal lipolysis and resynthesis pathways for triglycerides. After prodrug administration, neither L-Dopa nor dopamine was detected in the lymph, and peak lymph levels of the prodrug and related glycerides appeared 2 to 3 hours post dosing.

Differences in plasma L-Dopa and dopamine levels after L-Dopa and prodrug administration were not as dramatic as those observed in the lymph. Nevertheless, the temporal pattern of L-Dopa and the L-Dopa to dopamine ratio was very significantly different between the two modalities and a summary of the results is presented in Table 1. It is evident that the systemic bioavailability of L-Dopa in the rat (based on AUC data) is approximately doubled when administered as the prodrug rather than as the parent drug. Perhaps more significantly, the plasma levels of dopamine were significantly lower after prodrug administration than after administration of L-Dopa. For example, the ratio of the AUC values of dopamine to L-Dopa after L-Dopa administration was ≈ 3.9 whereas after administration of the prodrug the ratio was only ≈ 0.7. These data are consistent with the trends in the maximal plasma concentrations (Cp_{max}) of L-Dopa and dopamine.

The concentration profile of L-Dopa in the brain followed a similar temporal pattern to the plasma levels with the concentrations being approximately doubled after prodrug as compared to L-Dopa administration. The ratio of dopamine to L-Dopa was similar for the two modalities since the brain dopamine results from brain L-Dopa decarboxylase as systemic dopamine is

too polar to cross the blood brain barrier. The improved systemic and brain availability of L-Dopa and the lower dopamine to L-Dopa ratio after prodrug administration in the rat manifested itself in a very significant improvement in the pharmacologic/pharmacodynamic properties.[25]

The concept of glyceride prodrugs to help circumvent presystemic clearance follows from our understanding of how normal triglycerides are handled by the body, the details of which have been addressed in Chapter 2. Basically, triglycerides are not absorbed as such but must first be metabolized at the 1- and 3-positions by lipases to produce the symmetric monoglyceride and two fatty acids. The monoglyceride, due to its higher polarity, is absorbed into the enterocytes where the triglyceride is resynthesized through multiple steps and becomes an integral part of chylomicrons. Generally, drugs attached through an ester linkage to the 1- or 3-position of a triglyceride, or as a 1-monoglyceride, will be rapidly released by the action of lipase and this can lead to improved or altered delivery of the drug to the systemic circulation.[26,27] Few examples exist where substantial lymph delivery has resulted from such a modification, although an interesting example of an exception exists for the experimental hypolipidemic agent LK-A and its 1-monoglyceride, LK-903 (see Section V B).[28–30]

When drugs are attached to the 2-position of the triglyceride, as in the L-Dopa case, it is possible for the triglyceride to be a substrate for lipase. Lipolysis would produce the 2-monoglyceride prodrug which, once absorbed by the enterocyte, could be resynthesized to a new triglyceride. Whether the 2-monoglyceride would be a substrate for the resynthesis pathway would probably depend on its inherent physicochemical properties. One could speculate that the more the monoglyceride looks like a normal substrate, the more likely it is that it would be a suitable substrate.

The approach for preparing glyceride prodrugs in an attempt to circumvent presystemic metabolism appears to be meritorious, although it suffers from a number of limitations. Only drugs having a carboxylic group could be directly linked to one of the hydroxyls of glycerol, although spacer groups could be used to link an agent without a carboxyl group.[26] It would also depend on whether the triglyceride prodrug undergoes lipolysis without premature release of the drug in the lumen, is a substrate for the triglyceride resynthesis pathways and, on release to systemic circulation via the lymph, is capable of quantitatively releasing drug via systemic lipolysis. Of course, this should not take place in the liver.

III. PRODRUGS THAT TARGET THE LYMPHATICS

The selective delivery of anticancer drugs to the lymph system would be valuable in those instances where tumor cells spread via the lymphatics or metastasize within the lymph nodes. Similarly, the delivery of other chemotherapeutic agents to the lymph could prove useful in the treatment of bacterial

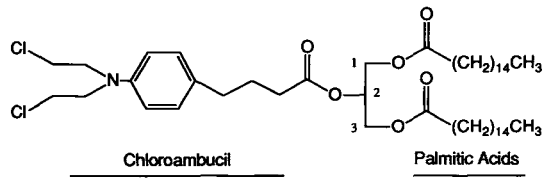


FIGURE 7. Chemical structure of a glyceride derivative of chlorambucil, where the chlorambucil occupies the 2-position and palmitic acid the 1- and 3-positions of the triglyceride.

and viral infections. The selective lymph delivery of immunomodulators can also be rationalized.

A. CANCER CHEMOTHERAPY

Chlorambucil is an effective agent for the treatment of lymphomas and is administered orally. It contains a carboxylic acid functional group, making the compound amenable to ready chemical modification, e.g., as a glyceride. Garzon-Aburbeh et al.,[31] using similar chemistry to that for L-Dopa, prepared 1,3-palmitoyl-2-[4-(bis(2-chloroethyl)amino]benzenebutanoyl]-glycerol which is a glyceride derivative of chlorambucil with the parent compound occupying the 2-position, and palmitic acid the 1- and 3-positions of the "triglyceride" (Figure 7). After oral administration of chlorambucil and its analog to mice inoculated with P388 leukemic cells which had been implanted S.C. (this route of administration results in the tumor cells disseminating along the lymphatics), an increased life span of the mice and a reduced toxicity was observed with the glyceride analog compared to chlorambucil itself. When the two compounds were administered by the I.P. route there was no difference in efficacy between the drug and its analog.

In a subsequent study, the lymph and plasma concentrations were assessed after oral administration of either radiolabeled chlorambucil or the corresponding analog. After administration of chlorambucil, 3.4 ± 1.5% of the administered dose was recovered from intestinal lymph whereas 26.4% of the radioactivity was recovered in the lymph after administration of the analog. After administration of the analog, no radioactivity peak was detected for chlorambucil, whereas for chlorambucil the nature of the radioactivity was not defined. All the recovered activity appeared to be associated with a peak corresponding to the analog, although the assay procedure would not have detected homologs produced from the lipolysis of the palmitoyl acids and their replacement by endogenous fatty acids (see earlier discussion on the L-Dopa derivative). Peak lymph levels for the analog were ~ 300 nmol/ml and occurred ~ 3.5 hr post dosing. For chlorambucil, peak lymph levels occurred at ~ 1 hr post dosing and were ~ 22 nmol/ml. Plasma levels peaked ~ 1 hr post dosing with a Cp_{max} of ~ 40 nmol/ml after chlorambucil, and for the analog the peak plasma levels occurred ~ 4 hr post dosing and were quite low. These results, unlike those for

the L-Dopa case, suggest that the superior pharmacologic properties of the analog after oral dosing were due to the intact analog and consequently the derivative did not appear to behave as a prodrug of chlorambucil.[31]

Contrary to this conclusion are some earlier findings by Wall et al.,[32] who found that when an alkylating agent similar to chorambucil was attached to various very lipophilic steroidal molecules, antitumor and antileukemic activity was only noted for those derivatives where the alkylating agent was attached to the steroid via a reversible linkage. Examples were ester or heterocyclic ether linkages. As was observed with the chlorambucil derivatives, the activity of the prodrugs was sensitive to the route of administration with the oral route resulting in significantly better activity than the S.C. or I.P. routes. The activity of the derivatives was also sensitive to the vehicle used to administer the drug with higher activity observed when administered in a sesame oil vehicle vs. an aqueous suspension. These data are not inconsistent with these alkylating agents being absorbed, in part, via the intestinal lymphatics and perhaps behaving as prodrugs.

The chlorambucil[31] data and the work of Wall et al.[32] highlight the following problems in evaluating many prodrug and analog studies: (a) is the activity due to the intact drug or a metabolite; (b) how are the results influenced by the route of administration and the vehicles used; and (c) are unusual routes, such as lymph transport, responsible for some of the observations. Only properly designed pharmacokinetic/pharmacodynamic studies using sensitive and specific analytical procedures will help answer some of these potential ambiguities. An alternate prodrug approach which holds promise for the delivery of some chemotherapeutic agents administered via the parenteral route involves macromoleular prodrugs and this approach is reviewed in Chapter 8.

B. CHEMOTHERAPY

Few studies have been published on the targeting or directing of drugs, via prodrug modifications, of antiviral, antifungal or antibacterial agents to the lymph after oral or parenteral administration.

γ-Aminobutyric acid, an important mammalian inhibitory neurotransmitter, may also play a role in microfilariae.[33] The use of prodrugs to deliver γ-aminobutyric acid (GABA) and inhibitors of GABA transaminase, such as γ-vinyl-GABA, to the brain has been of interest for a number of years. Jacob et al.[34–36] have studied the brain uptake and pharmacological properties of glyceryl lipid esters of GABA and γ-vinyl-GABA. Additionally, a mixed glyceride consisting of linoleic acid in the 1-position, GABA in the 2-position, and γ-vinyl-GABA in the 3-position of the glycerol was studied. It is difficult to draw conclusions from these studies in terms of the absorption processes, as pharmacokinetic data were not provided, nor was it suggested that these agents were transported via the lymph.

A similar strategy has been proposed by Deverre et al.[37] for the lymphatic delivery of GABA for the treatment of filariae residing in lymph. They prepared 1,3-dipalmitoyl-2-(4-aminobutyryl)glycerol HCl as a putative diglyceride prodrug of GABA. Although only *in vitro* studies were reported, the suspected lymphotropic prodrug was more active against infective larvae and microfilariae than GABA alone. Deverre et al.[37] noted that GABA was formed slowly from the prodrug during their *in vitro* studies while Jacob et al.[35] provided some data suggesting that GABA could be enzymatically cleaved from the triglyceride esters in brain homogenates.

An interesting and provocative paper dealing with the delivery of chemotherapeutic agents to the lymph was published by Malek et al.[38] Lymph delivery was effected by combining streptomycin, neomycin, viomycin, and other antibiotics with various macromolecules having carboxylic, sulfonic, or phosphoric acid functional groups. They formed complexes, or macromolecular salts, and these were referred to as "antibiolymphins".[38] Although it might be suggested that salts should not be considered as prodrugs, others have argued the converse.[3] Dramatic differences in lymph node levels were observed between administration of the parent antibiotic and the antibiolymphins after either I.M., I.P., or intrapleural administration. The basis for the increased levels of parent drug present in the lymph nodes after administration as the macromolecular species was that they were drained from the interstitial space into the afferent lymph vessels and subsequently transported to the lymph nodes. It was somewhat surprising that the drugs remained associated with the anionic macromolecules which drained into the lymph, and perhaps the relatively high pK_a values and the polarity of the antibiotics chosen in this case contributed to the apparent success reported by Malek et al.[38] The anatomical basis for the lymphatic clearance of macromolecules from the interstitial space is described in Chapter 1 and further examples are presented in Chapter 8.

IV. MODULATED DRUG INPUT RATE

There are no examples available where modulated, delayed, or prolonged drug input into the body via the lymph has been specifically stated as a study objective. The prolonged release of drugs into systemic circulation after oral dosing has been suggested as a possible advantage of intestinal lymphatic delivery although this concept has not been evaluated.[39] Some of the pharmacological advantages seen with the L-Dopa glyceride prodrug may have been due to the delayed and prolonged delivery effected by the prodrug (Section II B).[25] Similarly, the prolonged drug release reported for the antibiolymphins[38] may have been due to lymph delivery as well as the complexes acting as depot dosage forms. Future research will have to decide whether prolonged release of drugs to the systemic circulation is possible via prodrug manipulations that encourage lymph transport.

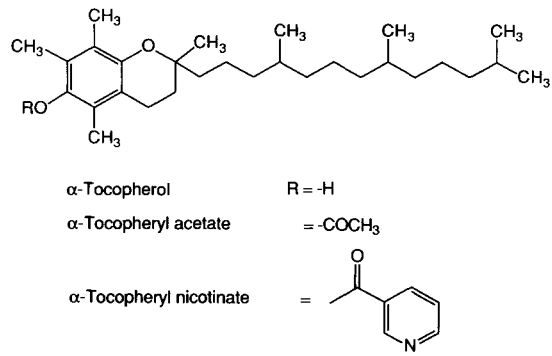


FIGURE 8. Structure of α-tocopherol and its acetate and nicotinate esters.

V. MISCELLANEOUS PRODRUG EXAMPLES

A. PRODRUGS OF LIPID SOLUBLE VITAMINS

The lipid soluble vitamins, vitamins A, D, E, and K are largely transported to the systemic circulation in the lymph. An extensive and excellent review of the literature on the absorption of these vitamins has been presented by Kuksis[40] and Chapter 4 also reviews some of the relevant literature. Prodrugs of the lipid soluble vitamins have been synthesized to improve their physicochemical stability and perhaps the degree to which the agents can be delivered to the systemic circulation.

1. Vitamin E

Vitamin E (a series of hindered phenols) acts as the body's antioxidant as well as having other more specific roles. Commercially, vitamin E is usually sold as α-tocopherol which readily undergoes oxidation and is therefore difficult to formulate for oral delivery. The acetate and nicotinate esters of α-tocopherol (Figure 8) have been extensively studied as prodrugs, although the nicotinate ester is also used as a peripheral vasodilator and therefore owes its activity to the release of nicotinic acid as well as α-tocopherol.

Since the site of oxidation in α-tocopherol is the phenol, blocking this group prevents the premature oxidation.[41] Nakamura et al.[41] prepared and evaluated a series of α-tocopheryl esters which included straight chain and branched alkyl esters, as well as succinic and aromatic esters such as the benzoate and nicotinate. Their principle objectives appeared to be the study of the hydrolysis kinetics of the α-tocopheryl esters in bile-pancreatic juice, small intestinal and liver homogenates of rats, as well as the cumulative lymphatic transport in thoracic duct fistulated rats after administration in an emulsion. It is assumed that the purpose of their work was to identify the most useful esters (prodrugs) for the delivery of α-tocopherol, even though the acetate[42] and

TABLE 2
Cumulative 12-hr Lymphatic Transport and the Composition of Various α-Tocopheryl-esters in Thoracic Duct Fistulated Rats After Gastric Administration as an Emulsion Formulation

Ester	Cumulative absorption in lymph (% of dose)[a]	Composition of radioactivity (%) as		
		Ester	α-Tocopherol	α-Tocopherol quinone
Acetate	52.3 ± 3.8	2	82	8
Palmitate	52.7 ± 5.5	2	57	16
Acid succinate	30.8 ± 2.9	2	75	8
Nicotinate	45.5 ± 3.2	9	55	15
O-hydroxybenzoate	9.6 ± 1.2	72	22	3
Pivalate	13.5	86	9	4

[a] Mean ± S.E.

Taken from Nakamura, T., Aoyama, Y., Fujita, L., and Katsui, G., *Lipids*, 10, 627, 1975. With permission.

succinate esters had been used as vitamin E sources, and that others had studied and evaluated the use of the nicotinate ester.[43,44]

The tocopheryl esters studied by Nakamura et al.[41] fell into a number of categories with respect to their lability in the presence of the bile-pancreatic fluid and the various tissue homogenates. The straight-chain esters were readily hydrolyzed whereas the aromatic esters were more slowly cleaved and the branched esters were poorly cleaved. In the *in vivo* lymphatic transport studies, the most readily hydrolyzed esters were transported as α-tocopherol, whereas the esters most resistant to pre-absorptive hydrolysis appeared unchanged in the lymph. However, the extent of the administered dose transported in the lymph was lower for the more slowly hydrolyzed esters than for the more readily hydrolyzed esters (Table 2). These transport data, when combined with the relative hydrolysis profiles, suggest that most of the α-tocopheryl esters were hydrolyzed prior to absorption even though intestinal hydrolysis of the α-tocopheryl esters may not be necessary for absorption.[41] The hydrolysis of α-tocopheryl acetate has been shown by Mathias et al. to occur by luminal enzymes, such as those associated with bile and pancreatic secretions, as well as intraluminal mucosal and intercellular enzymes.[45]

Significant differences were observed in the extent of tocopherol absorption from α-tocopheryl acetate between the studies of Nakamura et al.[41] and Gallo-Torres et al.,[43] and these differences demonstrate how experimental design[41] and vehicles[46] can influence the absorption and subsequent lymph transport of lipophilic materials. Fukui et al.[47] have attempted to optimize the absorption of *d*-α-tocopheryl acetate in rats and found that maximal absorption of α-tocopherol was from a medium-chain triglyceride/lecithin dispersed prepa-

TABLE 3
Summary of the 10-hr Post-Dosing Period After Oral Administration of α-Tocopheryl Nicotinate to Four Human Volunteers

Treatment	AUC^{0-10} (μg.hr/ml)	Relative bioavailability (AUC^{0-10}_{Fed} / AUC^{0-10}_{Fasted})	Cp_{max} (μg/ml)	t_{max} (hr)
200 mg				
Fasted	ND[a]		ND[a]	ND[a]
Fed	1.045 ± 0.274	∞	0.219 ± 0.047	6.0 ± 0.0
600 mg				
Fasted	0.068 ± 0.030		0.016 ± 0.005	6.0 ± 1.2
Fed	1.935 ± 0.460	28.5	0.506 ± 0.137	5.5 ± 0.5

Note: Area Under the plasma concentration versus time Curve (AUC, Mean ± S.E.M.), relative bioavailability and other relevant kinetic parameters summarized for 200 or 600 mg doses in volunteers in the fed and fasted states.

[a] ND = not detected

Taken from Hasegawa, J., Tomono, Y., Fujita, T., Sugiyama, K., and Mananura, K., *Int. J. Clin. Pharmacol. Ther. Tox.*, 19, 216, 1981. With permission.

ration. The extent of absorption was sensitive to the ratio of *d*-α-tocopheryl acetate to lecithin to medium chain triglyceride and was maximal when the ratio was 5:16:1. It appeared that the transport of α-tocopherol was mainly via the lymph as plasma levels were shown to minimally increase when the thoracic duct was fistulated, i.e., the lymph was drained. Consistent with this observation was the fact that Pluronic L-81, a potent inhibitor of the intestinal transport of chylomicrons,[48] significantly inhibited the lymph transport of α-tocopherol from the α-tocopherol medium-chain triglyceride/lecithin emulsion.[47]

The dramatic role that the intestinal environment can play on the absorption of α-tocopheryl esters is seen in the work of Hasegawa et al.[44] who studied the effect of food on the absorption of α-tocopheryl nicotinate in beagle dogs and human volunteers. Table 3 contains a summary of the human bioavailability data where up to a 29-fold increase in plasma levels was observed in subjects receiving the drug after food compared to the fasted state. In dogs, there was a very significant fivefold effect of food on bioavailability of the drug. Hasegawa et al. also reported a tenfold increase in the percentage of intact α-tocopheryl nicotinate transported in the lymph in fed vs. fasted rats, and when total α-tocopherol species (ester plus α-tocopherol itself) were analyzed, a twofold increase was observed between fed and fasted animals. These results point out the crucial role that dissolution rate may play in the absorption and subsequent lymph transport of highly lipophilic drugs from the gastrointestinal tract.

2. Vitamin A

Numerous chemical species make up the family of molecules displaying vitamin A activity; commercially available vitamin A is present as *trans*-retinol and various *trans*-retiny l esters such as retinyl acetate and retinyl palmitate. Ester derivatives have been utilized for formulation reasons rather than the promotion of lymphatic transport. For example, Forlano[49] prepared a series of mono- and di-α-substituted carboxylic acid esters of retinol with the expressed purpose of finding a more chemically stable form of vitamin A. Although many of the sterically hindered esters did have more desirable physicochemical properties than retinol, vitamin A palmitate showed the better vitamin A activity, followed by cod-liver oil, with only the α,α-dimethylpalmitate derivative having comparable vitamin A activity relative to the palmitate ester (71%).

Vitamin A has been shown to be absorbed from the gastrointestinal tract and to be transported to systemic circulation via the lymph.[50] Retinol recovered from lymph chylomicrons is present as retinyl esters,[51] with the palmitate ester the primary form.[52] Retinyl palmitate present in chylomicrons and intestinal VLDL stays associated with the lipoproteins in both *in vitro* and *in vivo* settings, and consequently this compound can be used as a marker for the disposition of chylomicrons and chylomicron remnants.[53] In an analogous manner to vitamin E, the intestinal absorption of retinyl esters is sensitive to formulation variables as well as whether bioavailability studies are conducted in the fed or fasted states. Dramatic food effects have been observed with retinoid drugs.[54] Noguchi et al.[55] have shown that lymphatic transport of vitamin A acetate in rats from emulsions was sensitive to the presence of the bile acid sodium taurocholate and egg phosphatidylcholine.

The overall role of dietary fat and polyunsaturated fatty acids on the absorption of vitamins A and their derivatives, as well as the other lipid soluble vitamins, has recently been reviewed by Kuksis.[40] After attempting to make sense out of the available literature, Kuksis[40] concluded that "... these studies indicate the general difficulties which arise in carrying out investigations on the intestinal uptake of fat-soluble vitamins and in comparing the various experimental absorption studies with each other." That is, the available literature only allows one to make some very tentative conclusions about the various factors that affect the absorption and lymph transport of the lipid-soluble vitamins and some of their prodrug derivatives.

B. LK-A AND LK-903

Reference to triglyceride and monoglyceride prodrugs/derivatives of hypolipidemic and hypocholesteric agents have appeared in the literature,[26–30,56] with the most interesting in terms of quantitative information relating to lymph delivery of the compounds LK-A and LK-903 (Figure 9).[28–30] Mizobe et al.[29] first evaluated the bioavailability of LK-A and LK-903 in beagle dogs.

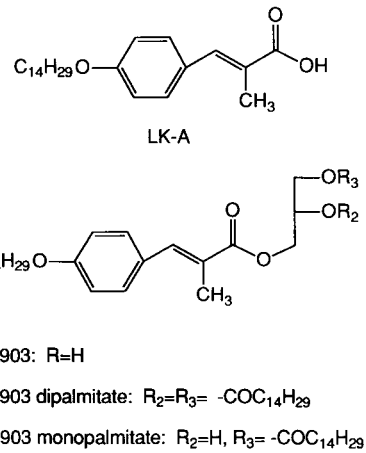


FIGURE 9. Structure of LKA, LK-903 and its mono- and di-palmitates.

Based on previous studies in their laboratories, LK-903 appeared to be a better hypolipidemic agent than LK-A even though the molecular basis for the hypolipidemic activity suggested that LK-A would be more active. It was concluded that perhaps the mode and extent of absorption may have influenced the conclusions from the pharmacological screening. In assessing the bioavailability of LK-A from an oil solution and LK-903 from a tablet formulation after oral administration, the plasma assays indicated four peaks by TLC scanning densitometry which corresponded to LK-A, LK-903, and surprisingly, the mono- and di-acylated forms of LK-903. The plasma profiles of LK-A and the related homologs are shown in Figure 10.

It is apparent that LK-903 breaks down, in part, to LK-A and is also converted to its mono- and di-acylated forms in which fatty acids such as palmitic acid are incorporated (Figure 10A). *In vitro* experiments showed a relatively slow breakdown of LK-903 to LK-A even though LK-903 is a 1-monoglyceride. When LK-A was administered in a traditional solid dosage form, no drug was detected and it was only after an oily solution was administered that plasma levels were detected.[29] From a qualitative standpoint, the plasma profiles were similar after administration of either LK-A or LK-903, although quantitatively, the levels of each species differed.

After intravenous administration of LK-A and LK-903, the respective mono- and di-acylated species were not observed and there did not appear to be interconversion between LK-A and LK-903. When the two acylated compounds were administered, the mono-acylated species formed LK-903 and a low level of LK-A. The di-acylated material sequentially produced the mono-acyl species and small quantities of LK-903 and LK-A.[29] The low level of LK-A which was formed is consistent with LK-903 and the mono- and di-acylated species being relatively poor substrates for lipolysis at the 1-acyl function.

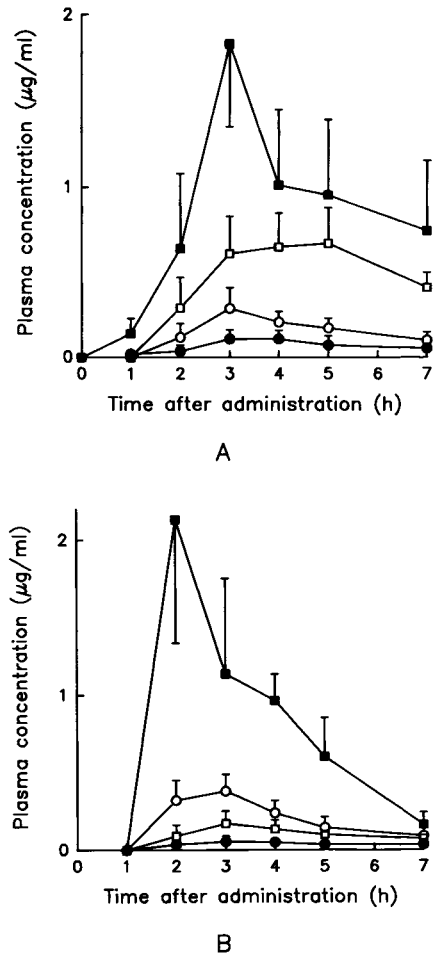


FIGURE 10. Plasma concentration versus time profiles for LK-903, LK-A, and its homologs after oral administration of (A) LK-903 from an experimental tablet, and (B) LK-A in an oil solution. Symbols: ●, LK-903; ○, LK-A; □, monoacylated LK-903; ■, diacylated LK-903. (From Mizobe, M., Matsuda, S., Yoneyama, T., and Kohno, K., *Chem Pharm. Bull*, 31, 2424, 1983. With permission).

Therefore, although there are some significant similarities between the processing of all these glyceride mimics and normal glycerides, the cleavage at the 1-position was somewhat atypical.

The bioavailabilities of LK-903 and LK-A estimated from the areas under the plasma concentration versus time curves (AUC) were very sensitive to dosage form effects (Table 4). A greater than threefold difference was observed in the relative bioavailability between the powder and the lecithin solution dosage forms of LK-903, while a greater than 40-fold difference was observed

TABLE 4
Summary of Oral Administration of LK–903 or LK-A to Beagle Dogs

Compound administered	AUC^{0-10} (μg.hr/ml)[a]	Relative Bioavailability ($AUC^{0-7}_{experimental}/AUC^{0-7}_{powder}$)
LK-903		
Powder	7	1.0
Tablet	8.5	1.21
Lecithin mixture	11	1.57
Emulsion	17.5	2.50
Lecithin solution	23.5	3.36
LK-A		
Powder	≈ 0.5	1.0
Tablet	≈ 0.75	1.5
Oil suspension	2.25	4.5
Oil solution	7.5	15
Lecithin mixture	13	26
Lecithin solution	21	42

Note: Summary of Area Under the plasma concentration versus time Curve (AUC) and relative bioavailability after oral administration from various dosage forms.

[a] Data are estimated from figure 7 in reference 29.

Taken from Mizobe, M., Matsuda, S., Yoneyama, T., and Kohno, K., *Chem. Pharm. Bull.*, 31, 2424, 1983. With permission.

with LK-A. Due to the similarity between the processing of normal long chain fatty acid triglycerides and LK-903 and LK-A, Mizobe et al. proposed the scheme shown in Figure 11 to account for their observations.

The implication in Figure 11 that both LK-903 and LK-A are transported to the systemic circulation largely as di-acylated LK-903 via the lymph was confirmed in the later study by Sugihara and Furuuchi.[30] When LK-903, radiolabeled with ^{14}C in the glycerol portion of the molecule or by ^{3}H in the cinnamic acid portion of the molecule, was orally administered to thoracic lymph duct cannulated rats as a suspension in 0.1% hydrogenated castor oil, 18.1% and 31.1% of the radioactivity was recovered from the lymph over 24 hr, respectively. The majority of the radioactivity was with the triglyceride analogues or the di-acylated LK-903 portion of the TLC-autoradiogram (60 to 70% after ^{14}C-LK-903; 50 to 80% after ^{3}H-LK-903). It appeared that ^{14}C-glycerol released from LK-903 due to some conversion to LK-A was not transported in the lymph.

When ^{3}H-LK-A was orally administered to rats, 6.7% of the dose appeared in the lymph over a 24-hr period. Depending on the sampling time, 3 to 13% of the the radioactivity was recovered as LK-A, 4 to 8% as LK-903, 13 to 19% as mono-acylated LK-903 (diglyceride equivalent), and 61 to 81% as di-

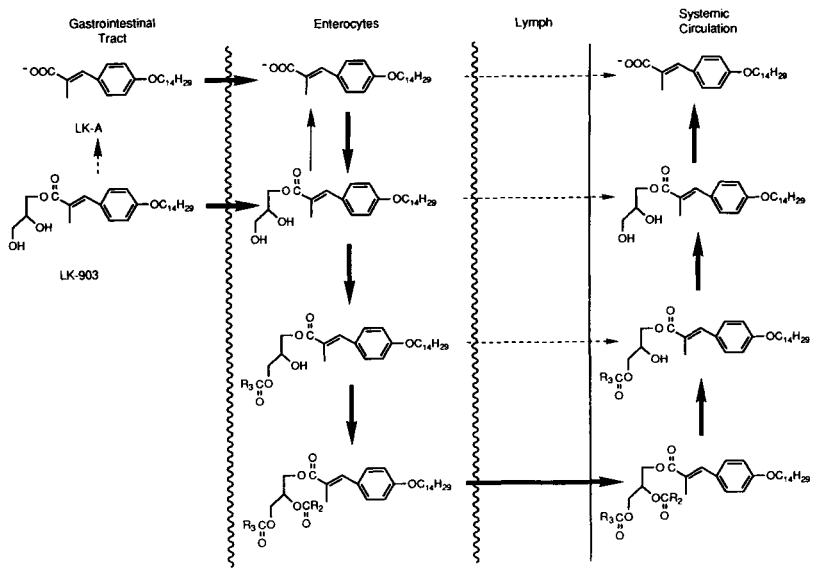


FIGURE 11. Mechanism proposed for the absorption and intestinal lymphatic transport of LK-903 and LK-A. (From Sugihara, J. and Furuuchi, S., *J. Pharmacobio-Dyn.*, 11, 121, 1988. With permission).

acylated LK-903 (triglyceride equivalent). Doubly-labeled material was also administered and the observations were similar to those made with the singly-labeled materials.

Sugihara and Furuuchi[30] also examined the effects of various vehicles on the lymphatic absorption of LK-A and LK-903 and observed a rather dramatic increase in lymph delivery when lecithin was used in place of the hydrogenated castor oil used in the experiments described earlier. The results are presented in Table 5. It was uncertain whether the increase in bioavailability of LK-A from 6.7% to 47.9% was due to specific effects on lymphatic transport or an overall increase in bioavailability. The bioavailability data from Mizobe et al.[29] strongly suggested that the increase was due to a bioavailability effect and, therefore, the increased bioavailability also resulted in an improvement of lymphatic drug transport.

The results from these studies showed that the absorption and biochemical processing of LK-A and LK-903 were similar to those for natural fatty acids and monoglycerides except for the fact that LK-903 as a monoglyceride was poorly converted to LK-A. This was confirmed with other agents by Sugihara et al. in a separate study.[26] As stated by Mizobe et al.,[29] these "findings may give some insight into the design and preformulation study of new drugs having structures analogous to those of natural fatty acid glycerides."

TABLE 5
Effect of Different Pharmaceutical Vehicles on the Lymphatic Transport of LK-A and LK-903 in the Rat

Compound (dose)	Dosage form	% of dose transported via lymphatics in 24 hr
^{3}H-LK-A (83.5 mg/kg)	0.1% HCO[a]	6.7 ± 1.0 (n = 3)[b]
	Lecithin (170 mg/kg)	47.9 ± 6.4 (n = 4)
3H-LK-903 (100 mg/kg)	0.1% HCO (10 ml/kg)	31.1 ± 5.8 (n = 3)
	O.1% HCO + triolein (0.5 g/kg)	20.8 ± 4.2 (n = 3)
	O.1% HCO + triolein (3 g/kg)	21.7 (n = 2)
	Lecithin (170 mg/kg)	41.4 ± 3.5 (n = 4)

[a] Hydrogenated castor oil (Nikkol).
[b] Mean ± S.E.

Taken from Sugihara, J. and Furuuchi, S., *J. Pharmacobio-Dyn.*, 11, 121, 1988. With permission.

C. ESTER AND GLYCERIDE PRODRUGS TO DECREASE THE GASTROINTESTINAL IRRITATION OF NONSTEROIDAL ANTI-INFLAMMATORY AGENTS

Many nonsteroidal anti-inflammatory agents, including indomethacin, have severe gastrointestinal side-effects, and various efforts at reducing these effects through prodrugs have had some success. Kobayashi et al.[57] and Mishima et al.[58] evaluated the farnesyl ester of indomethacin (Figure 12) for its ability to systemically deliver indomethacin while also evaluating its lymph delivery.

Twelve percent of radiolabeled indomethacin farnesil in sesame oil orally administered to rats was transported via thoracic lymph, and greater than 90% of the radioactivity was present as the intact ester with the balance being indomethacin. The ester was found to be a substrate for rat pancreatic and plasma enzymes, but a relatively poor substrate for pancreatic enzymes in the dog and man, and the plasma enzymes in the rabbit, dog, monkey, and man. The ester was more susceptible to cleavage in edematous rat paw homogenates than in homogenates of normal rat paws.

It appeared that the majority of the ester which reached the systemic circulation was transported lymphatically, although drug concentrations in rat paws, blood, and other tissues suggested that the ester did not quantitatively deliver indomethacin to the systemic circulation. However, the ester did produce some interesting changes in indomethacin distribution when compared to the administration of indomethacin. No data were presented on the degree of gastrointestinal side-effects between the ester and indomethacin itself.

A number of glyceride prodrugs of indomethacin have also been evaluated by Paris et al.[59] who found that three derivatives (indomethacin-1-monoglyceride, indomethacin-2-monoglyceride and the 1,3-dilaurylglyceryl

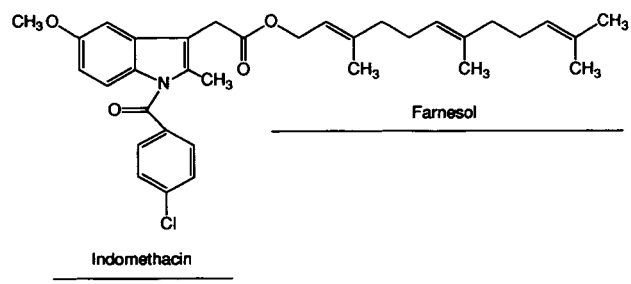


FIGURE 12. Chemical structure of the farnesyl ester of indomethacin.

ester) produced lower but comparable anti-inflammatory activity to indomethacin using the rat adjuvant arthritis model. Additionally, indomethacin-2-monoglyceride and the 1,3-dilaurylglyceryl ester of indomethacin appeared to produce significantly less acute gastric irritation than indomethacin, thereby producing a potentially better therapeutic index. Similar studies have been performed on various glyceride prodrugs of aspirin[60–63] and other anti-inflammatory agents,[26,64] although only the work of Kumar and Billimoria[61] and Sugihara et al.[26] attempted to estimate the extent of lymphatic transport. In the other studies, the rationale for the preparation of the glycerides was to promote lymph delivery, although experimental verification was not attempted. The glycerides showed some interesting activities and, in general, showed a lower degree of gastric irritation when compared to the parent compounds.

D. OTHER PRODRUGS

Attempts have been made to attach drugs such as certain β-blockers to various glyceride-like groups,[65,66] and the resulting amphiphilic molecules have been shown to spontaneously disperse. They have been labeled "pharmacosomes" because of their tendencies to form unilamellar vesicles. Although it has been suggested that these molecules should enhance lymph transport, no experiments confirming lymph selectivity have been presented.

Lipophilic esters of various neuroleptic agents have been used and tested as depot dosage forms.[1–3] Matsunaga et al.[67] have recently shown that radiolabeled haloperidol decanoate in sesame oil administered intramuscularly results in very significant levels of radioactivity in lymph nodes nearest to the injection site. No regional lymph node uptake was seen with intravenously administered drug. Although aspects of the behavior of the depot antineuroleptic agents may be due to their lymph transport, Matsunaga et al.[67] demonstrated that brain uptake of haloperidol from haloperidol decanoate was only associated with the systemically-released haloperidol.

These results suggest the possibility that fatty acid esters of anticancer agents or other drugs might be used to target the lymph nodes.

VI. CONCLUSIONS AND PERSPECTIVES

Lymph transport is a highly complex biochemical and transport process, especially after oral dosing. In this chapter, an attempt was made to review the literature pertinent to the use of prodrugs to effect lymph delivery after oral administration. None of the examples described fully achieved the goal of quantitative delivery via the lymph, although a number of studies were very informative. It appears that we will have to be "smarter" than in the past if we truly wish to manipulate lymph transport via the prodrug approach. Simply making more lipophilic prodrugs through a simple esterification process, or even glyceride formation, is not likely to be fully successful due to the problems of metabolic stability.

To achieve better systemic delivery of orally administered compounds via the intestinal lymphatics, we will have to:

1. Better understand and perhaps more effectively utilize the complex biochemical and transport processes that affect the lymph transport of natural substances such as the lipid soluble vitamins, cholesterol, and normal lipids
2. Perform basic research into the design of promoieties and linkages that cause changes in drug lipophilicity and are also selectively-cleaved post absorption. Specifically, promoieties will have to be designed that are metabolically and chemically stable during the absorption and transport phases of delivery to the lymph and undergo conversion selectively at sites other than the liver

For the selective delivery of chemotherapeutic agents (as prodrugs) to the lymph after oral dosing, we may have to more fully utilize our knowledge of the enzymes present in lymph.[68] Selective reversion in the lymph and at the lymph nodes may prove necessary for selective delivery. A high degree of lipophilicity in terms of both partitioning and lipid solubility, luminal and enterocyte metabolic stability, selective chemical or biochemical reversion along with the ability to be delivered to the absorbing surface from the dosage form, appear necessary for effective lymphatic transport after oral administration.

For parenteral drug delivery to lymph as prodrugs, especially macromolecular prodrugs, the site of administration, regional selectivity, and the effect of molecular size appear to be critical factors.[69,70] Chapter 8 addresses other aspects of lymph delivery from parenteral dosage forms.

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Chapter 6

THE CLEARANCE OF LIPOPROTEINS AND OF DRUGS ASSOCIATED WITH LIPOPROTEINS

Tetsuo Noguchi

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I. LIPOPROTEINS: CARRIERS FOR LYMPHATIC TRANSPORT OF LIPOPHILIC DRUGS

Drugs that are absorbed and transported through the lymphatic system drain, via the thoracic lymph duct, into the systemic circulation at the junction of the jugular and left subclavian veins (see Chapter 1 for details). Drug molecules with varying degrees of hydrophilicity and lipophilicity can be transported via the lymphatic system, although the extent of transport depends on factors such as the site of administration, physicochemical properties of the drug, dose administered and various vehicle effects. These various issues are described elsewhere in this book.

The lymphatic transport of various lipophilic xenobiotics has been systematically studied by Sieber[1] and it was concluded that lipophilicity is a key determinant of the extent of intestinal lymphatic transport after oral administration. Metabolic transformation during the absorption and transport processes which may decrease the effective lipophilicity of the drug can also affect the distribution of these compounds within the lipid and aqueous fractions of lymph.[2] Various studies which have been conducted regarding the mechanisms of intestinal lymphatic transport of numerous compounds after oral administration[3–9] have indicated that lipophilicity, as estimated by partition coefficient data and/or lipid solubility, is a major determining factor of the extent of transport. As described in Chapters 3 and 4, the mechanisms and the extrinsic factors determining the extent of lymph transport of lipophilic compounds are complex and the controlling factors are an area of ongoing research. It is clear, however, that the lipoproteins formed by the intestinal enterocyte play a fundamental role in the transport of orally administered lipophilic compounds.

This chapter will review the fate of lipophilic compounds once they have been transported through the lymphatic system, i.e., how is the clearance of drugs, especially those associated with the chylomicron fraction of lymph, affected by the association with the lipoproteins? Furthermore, the clearance of lipophilic drugs and xenobiotics transported and distributed via lipoproteins will be addressed in relation to their inherent clearance as well as the clearance of the lipoprotein carriers.

It is well known that the primary transport carriers of lipophilic compounds in the intestinal lymph are the chylomicrons and very low density lipoproteins (VLDL).[10,11] Lipophilic pollutants such as benzo(a)pyrene,[12] 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin,[13] DDT,[14] toxoids such as aflatoxin and its palmitate ester,[15] and some steroids[16] are transported by the lymphatics in association with chylomicrons and/or the VLDL fraction. The nature of drug/lipoprotein clearance is highly complex and much of the progress made in understanding lipoprotein clearance has come from research conducted into atherosclerosis.[17] An example of the complexity of drug/lipoprotein clearance is the metabolic fate of orally administered α-tocopherol. McCormick and coworkers[18] determined the distribution of α-tocopherol among serum lipopro-

teins after oral administration and found that the administered drug, although initially associated with the chylomicron and VLDL fractions of the lymph, was then distributed to the low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions as a function of time. The clearance of drugs associated with lipoproteins has become of major concern for those interested in drug delivery research. The potential role of lipoproteins as carrier systems for therapeutic agents has been recently reviewed.[18a] The following section briefly describes the metabolism and clearance kinetics of carrier lipoproteins and associated drugs.

II. FATE OF LIPOPROTEINS IN THE SYSTEMIC CIRCULATION

A. THE SIGNIFICANCE OF LIPOPROTEIN METABOLISM

Chylomicrons are synthesized in the epithelial cells of the small intestine and enter the systemic circulation through the lymphatics. Chylomicron associated triglycerides are metabolized in extrahepatic capillary beds where the majority of the triglyceride is removed by lipoprotein lipase.[19] As a result of this initial metabolism, chylomicrons are converted from triglyceride-rich to cholesteryl ester-rich particles which are termed chylomicron remnants. The remnants are subsequently removed from the plasma by the liver through receptor-mediated processes which are dependent upon the recognition of specific surface apolipoproteins.[20,21] Histological studies provide evidence that the internalization and the subsequent degradation of chylomicron remnants, as well as low-density lipoproteins (LDL), follow the classical receptor mediated endocytosis pathway.[22]

Vost[23] examined the uptake of circulating chylomicron triglyceride by using a perfused intact rabbit aortic wall preparation and assessed the role of triglyceride hydrolysis on the uptake process. It was determined that the uptake of chylomicron triglyceride by perfused aorta was largely independent of the initial triglyceride hydrolysis.

Phospholipids, which are a major surface component of chylomicrons, are lost during interaction with lipoprotein lipase. The surface phospholipids are transferred to the HDL fraction[24] and have an important role in HDL formation and cholesterol homeostasis. The interrelationships between the different lipoprotein classes in terms of their metabolism and clearance are represented schematically in Figure 1.

B. KINETICS OF LIPOPROTEIN CLEARANCE

Chylomicron metabolism is rapid and complex, with multiple interactions occurring with different enzyme systems, different apolipoprotein classes, plasma lipoproteins, and cell receptors. There have been a number of different mathematical models proposed for describing the clearance of lipoproteins. For example, a model describing the kinetics of lipoprotein removal from the

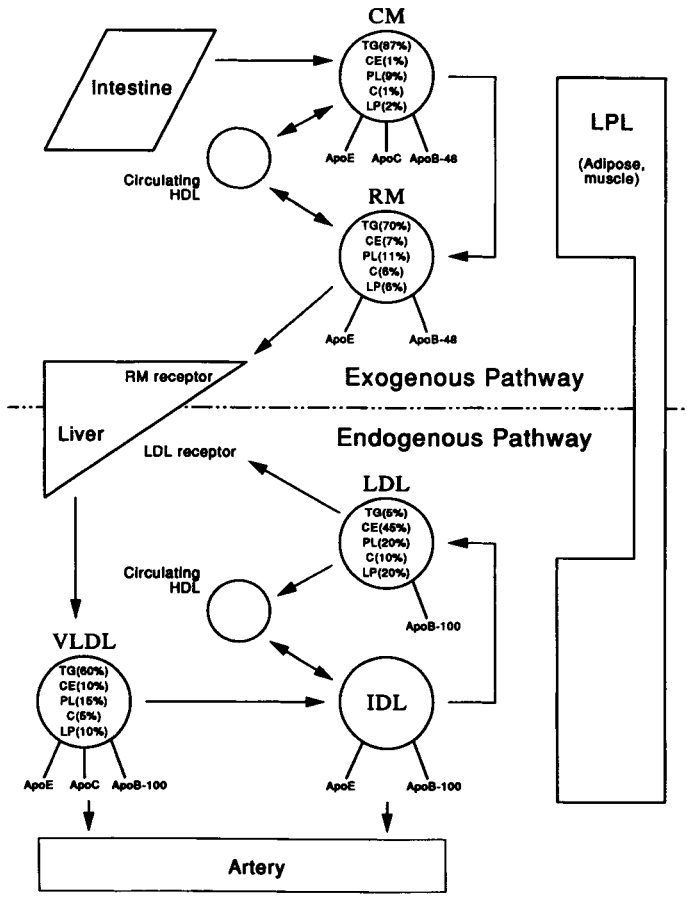

FIGURE 1. Schematic representation of lipoprotein transport in man. Only those apoproteins that play a role in receptor-mediated transport are shown and the approximate composition of each lipoprotein is listed. Abbreviations are as follows: LPL, lipoprotein lipase; CM, chylomicrons; RM, chylomicron remnants; VLDL, very-low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; TG, triglyceride; CE, cholesteryl ester; PL, phospholipids; C, cholesterol.

plasma has been developed by Harris and co-workers after investigating chylomicron metabolism after vascular injection.[26–28] Foster and Berman[29] proposed a kinetic model describing the *in vitro* hydrolysis of rat chylomicron acylglycerols. However, this particular model did not take into account the receptor-mediated removal of chylomicron remnants (containing residual triglycerides) which contribute to the overall kinetics of lipoprotein metabolism.

Recently, Redgrave and Zech[30] assessed the contribution of chylomicron remnant removal to triglyceride hydrolysis kinetics by simultaneously

tracing the plasma removal of chylomicron cholesteryl esters and chylomicron triglycerides. In their studies, they compared the metabolism of intravenously administered radiolabeled chylomicrons in three groups of rats. The radiolabeled chylomicrons were prepared by administering [1-^{14}C]palmitic acid as a triglyceride precursor and [1,2(n)-^{3}H]cholesterol as the cholesteryl ester precursor to donor rats, collecting the lymph and then administering it to the rats in the different study groups. In their study, one group of rats was starved, and the other two groups were fed single meals which contained either 5% fat or were fat-free.

The different nutritional states of the recipient rats altered the pattern of metabolism of the injected chylomicrons. However, in each study group the clearance of triglyceride was more rapid than cholesteryl ester removal. These data are consistent with previous findings and with the sequential removal of triglycerides by lipoprotein lipase-mediated hydrolysis followed by hepatic uptake of remnant particles.[25] The recovery of radioactivity associated with the administered cholesteryl esters in the liver 30 min. after injection was similar in all treatment groups at approximately 20% of the injected dose. Hepatic recovery of triglyceride associated radioactivity was approximately 5% of the administered dose in the groups of fed rats, and slightly less for the fasted rats.

Using a multicompartmental model to describe chylomicron core lipid metabolism, Redgrave and Zech[30] derived various kinetic parameters describing the clearance of the chylomicron triglycerides and cholesteryl esters. In their kinetic model they introduced chains of compartments, T1-T5 and C1-C5, which described the clearance of chylomicron triglyceride and cholesteryl ester, respectively. The turnover times for each compartment, in either chain, were constrained to be equal as were the chylomicron remnant pool turnover times for either lipid. This feature of the model recognized that some chylomicrons need little triglyceride loss before removal as a particle. Chylomicron cholesteryl ester removal from the plasma represented uptake of remnant particles, whereas triglyceride removal represented the sum of remnant particle uptake and lipoprotein lipase-based hydrolysis. The clearance and turnover data for chylomicron triglyceride and cholesteryl ester determined from this kinetic model are consistent with values reported in humans.[31,32] A "delipidation index" of remnant particles was calculated from the ratio of chylomicron cholesteryl ester and triglyceride residence times. The delipidation index was positively correlated with the residence time of the remnant, and significant differences existed in the delipidation index between the starved group and both normal-fed and fat-free-fed groups. For example, compared with rats fed a meal of the standard 5% fat diet, the starved rats exhibited more extensive delipidation. A longer residence time of chylomicron triglyceride in rats fed a fat-free meal was explained by less extensive delipidation, although remnant removal appeared to be accelerated. More extensive studies are necessary to clarify the effect of fasting on remnant clearance. Table 1 lists the kinetic

parameters derived from this study. The utility of these kinetic models lies with the potential for modeling the clearance of lipoprotein lipid and for discriminating between the different processes involved with lipoprotein metabolism.

C. FACTORS AFFECTING LIPOPROTEIN CLEARANCE

The hepatic metabolism of lipoproteins has been extensively studied, particularly in relation to atherosclerosis. Many factors are thought to modulate and lead to changes in the hepatic secretion rate of VLDL. Stress, caffeine, smoking, diabetes, obesity, and the size and frequency of meals are thought to be major factors leading to atherosclerosis. However, evidence and data implicating any one factor have been equivocal since the different factors could be operating simultaneously and contributing either a positive or negative effect.[18] Under such circumstances, kinetic studies are sometimes useful in removing the potential ambiguity.

For example, the effect of contraceptive steroid use by young, healthy women on the hepatic uptake of chylomicron remnants was studied by determining the plasma decay (concentration-time profile) of retinyl palmitate-labeled chylomicrons in parallel groups of women either on or off contraceptive steroid therapy.[31] The plasma clearance rates of retinyl palmitate-labeled chylomicrons was significantly increased during contraceptive steroid uptake (74 vs. 115 ml/min) indicating enhanced hepatic uptake of chylomicron remnants and an increased hepatic uptake of higher density lipoproteins.

Phosphatidylcholine, which is a major surface component of chylomicrons, is lost from chylomicrons during remnant formation and remnant uptake by the liver. It is reasonable to speculate that the nature of chylomicron phosphatidylcholine could influence the overall rate of chylomicron clearance. Robins and co-workers[32] intravenously administered different model chylomicrons to unanaesthetized rats and determined the clearance of emulsion lipids. As model chylomicrons, emulsions were prepared with free ^{14}C-cholesterol, ^{3}H-cholesteryl oleate, triolein, and different phospholipids with differing fatty acid substitution patterns (i.e., different hydrophilicity). The effect of the different phospholipids on emulsion lipid clearance is presented in Table 2. The rate of emulsion lipid clearance was closely correlated with the hydrophilic strength (fatty acid chain length) of the phospholipids present on the surface of the emulsion particle. The cholesteryl ester and triolein clearance data suggest that the process of remnant uptake and remnant formation were both influenced by the specific phospholipid composition on the emulsion particle surface.

The effect of lipid saturation on the clearance of plasma chylomicrons was studied by Renner and co-workers[33] and the relevant data are presented in Table 3. They characterized lymph lipoproteins of $d < 1.006$ g/ml, and found that despite the similarity of size between chylomicrons formed from saturated and unsaturated triglyceride, the apoB content of unsaturated linoleate chylo-

TABLE 1
Clearance and Residence Time Characteristics of Various Chylomicron Components in Rats

	Parameter	Units	Fasting	Chow-fed	Low-fat
A	Residence time of individual compartments TX[a] and CX[b]	Min	2.03 ± 0.30	1.86 ± 0.42	2.23 ± 0.37
A′	Fractional rate of exit from compartments TX and CX	Min^{-1}	0.524 ± 0.30	0.613 ± 0.42	0.508 ± 0.37
B	Fractions of TX and CX taken up as particles		0.187 ± 0.46	0.247 ± 0.43	0.242 ± 0.34
C	Fractions of the remaining TX which are delipidated		0.409 ± 0.12	0.387 ± 0.30	0.326 ± 0.43
D	Residence time of RM[c]	Min	11.2 ± 0.43	9.27 ± 0.49	7.33 ± 0.49
E	Residence time of chylomicron triglyceride	Min	4.15 ± 0.33	3.73 ± 0.29	4.68 ± 0.36
F	Residence time of cholesteryl ester	Min	10.9 ± 0.29	8.14 ± 0.30	8.38 ± 0.36
G	Mean index of delipidation		2.73 ± 0.20	2.21 ± 0.17[d]	1.89 ± 0.28[e]

Note: Kinetic, model-dependent parameters (mean of individual value ± fractional standard deviation) in 10 rats studied per group. Characteristics of chylomicron components after administration of radiolabeled chylomicrons to rats which were either fasted, fed a high-fat (chow-fed) meal, or low fat meal.

[a] TX = plasma triglyceride compartment.
[b] CX = plasma cholesteryl ester compartment.
[c] RM = chylomicron remnant pool.
[d] Significantly less than fasting, $P < 0.025$
[e] Significantly less than fasting, $P < 0.01$

Taken from Redgrave, T. G. and Zech, L. A., *J. Lipid Res.*, 28, 473, 1987. With permission.

TABLE 2
Effect of Phosphatidylcholines on Rate of Emulsion Lipids Clearance

Emulsion PC[a] administered	Fractional Clearance (min^{-1})[b]			
	Emulsion PC	FC	CE	Triolein
20:1 – 20:1	0.038 ± 0.014	0.020 ± 0.006	0.012 ± 0.005	0.229 ± 0.061
18:0 – 18:2	0.058 ± 0.028	0.089 ± 0.025	0.077 ± 0.026	0.380 ± 0.046
16:0 – 18:2	0.079 ± 0.040	0.113 ± 0.026	0.121 ± 0.032	0.467 ± 0.249
16:1 – 16:1	0.092 ± 0.025	0.125 ± 0.013	0.124 ± 0.033	0.542 ± 0.105

Note: Rate of clearance of emulsion lipids after intravenous administration to rats. PC = phosphatidylcholine; FC = free cholesterol; CE = cholesteryl ester.

[a] The PC are listed in increasing order of hydrophilic strength.

[b] Calculated from the serum disappearance curves. Data are mean ± SD (n = 4 – 6 per group).

Taken from Robins, S. J., Fasulo, J. M., and Patton, G. M., *J. Lipid Res.*, 29, 1195, 1988. With permission.

TABLE 3
Relationship Between Components of Mesenteric Chylomicrons and VLDL During Steady-State Lipid Absorption

	N[a]	ApoA-I/TG	ApoB/TG	PL/TG	(ApoA-I + ApoB + PL)/TG
Chylomicrons					
Linoleate	7	0.004 ± 0.001	0.008 ± 0.001[b]	0.088 ± 0.009	0.099 ± 0.008[b]
Palmitate	7	0.005 ± 0.002	0.016 ± 0.003[b]	0.137 ± 0.021	0.160 ± 0.022[b]
VLDL					
Linoleate	4	0.018 ± 0.004	0.044 ± 0.015	0.388 ± 0.111	0.450 ± 0.125
Palmitate	4	0.022 ± 0.006	0.056 ± 0.024	0.291 ± 0.004	0.369 ± 0.019

Note: Whole lymph collected during steady-state lipid absorption of either palmitate or linoleate was separated into chylomicron and VLDL fractions by ultracentrifugation at 27°C. Ratios of the major surface (apoA-I, aopB, PL) and core (TG) components were calculated.

[a] N = number of experiments, data presented as mean ± S.E.M.

[b] Statistically significant differences between groups calculated by analysis of variance, $P < 0.05$.

Taken from Renner, F., Samuelson, A., Rogers, M., and Glickman, R. M., *J. Lipid Res.*, 27, 72, 1986. With permission.

microns was significantly lower than that of palmitate chylomicrons. However, this difference did not appear to alter their relative plasma clearance profiles. In these same studies, the clearance of palmitate chylomicrons from the circulation of recipient rats was only slightly more rapid than that of linoleate chylomicrons ($t^1/_2$ = 6.3 vs. 6.6 min).

Changes in lipoprotein metabolism associated with diabetes have been extensively studied to determine how the changes may influence the development of cardiovascular disease associated with diabetes. Lipoprotein metabolism and clearance in the two major forms of human diabetes, noninsulin-dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) was recently reviewed by Howard.[34] There appeared to be multiple changes in lipoprotein metabolism in both NIDDM and IDDM. However, the mechanism of these changes, the sequence in which they occurred, and in particular, their relevance to the atherosclerotic process are yet to be fully elucidated.

Clearance rates for chylomicrons have been measured in patients with normal plasma triglyceride levels or with endogenous hypertriglyceridemia.[35] Patients with normal triglyceride levels exhibited a rapid chylomicron clearance rate ($t^1/_2$ = 4.5 min) whereas clearance in patients with hypertriglyceridemia was prolonged ($t^1/_2$ = 23 min). More recently, the kinetics of chylomicron remnant clearance was studied in hyperlipoproteinemic and hypertriglyceridemic subjects.[36] In this study, the chylomicron core lipid was labeled with retinyl esters and the chylomicron remnant clearance in normal subjects (Group A) was compared with that in hypertriglyceridemic (Group B) and hyperlipoproteinemic subjects (Group C). From the multicompartmental kinetic analysis performed on the data, the respective half-lives of remnant and chylomicron clearance were 14.1 and 16.7 min in Group A, 50.7 and 42.5 min in Group B, and 611.9 and 69.8 min for Type 3 subjects in Group C. It was suggested that delayed chylomicron remnant clearance observed in subjects with endogenous hypertriglyceridemia might be a consequence of an overproduction of VLDL where the VLDL remnants may compete with chylomicron remnants for removal by the liver via apoE receptor-mediated endocytosis.

In summary, many factors such as diet, disease, and the characteristics of the core and surface of the lipoprotein appear to affect their clearance. The obviously complex mechanisms should, however, be clarified by quantitative studies of the various components and metabolic pathways along with appropriately adapted pharmacokinetic models.

III. CLEARANCE OF DRUGS ASSOCIATED WITH LIPOPROTEINS IN THE SYSTEMIC CIRCULATION

A. LIPID SOLUBLE VITAMINS

Many studies have been conducted into the lymphatic transport of the lipophilic vitamins such as retinol, cholecalciferol (vitamin D_3), and α-toco-

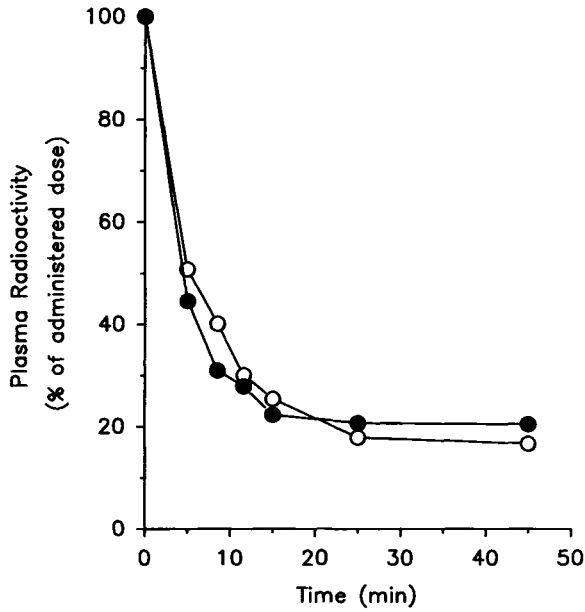

FIGURE 2. Clearance of chylomicron [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3 from plasma. Half a milliliter of labeled lymph (0.29 μCi of [^{3}H]retinol (●) and 0.02 μCi of ^{14}C-labeled vitamin D-3 (○)) containing 9.2 mg triacylglycerol was injected intravenously into rats and radioactivity in plasma was determined as a function of time. (From Blomhoff, R., Helgerud, P., Dueland, S., Berg, T, Pedersen, J. I., Norum, K. R., and Drevon, C. A., *Biochim. Biophys. Acta*, 772, 109, 1984. With permission).

pherol (vitamin E). For example, Blomhoff and co-workers[37] studied the lymphatic uptake and transport of retinol and cholecalciferol from rat intestine and confirmed that both vitamins were transported via the intestinal lymphatics in association with chylomicrons. They observed that almost all the transported retinol was present as retinyl ester whereas the cholecalciferol appeared in chylomicrons as such. In terms of systemic clearance, it was determined that the retinyl esters were a relatively nonexchangeable component of chylomicrons and their remnants, while significant amounts of cholecalciferol were transferred from chylomicrons to other plasma fractions.

Blomhoff and co-workers[37] compared the plasma clearance of chylomicron ^{14}C-cholecalciferol with chylomicron ^{3}H-retinyl ester by injecting double-labeled lymph intravenously into rats. The resulting clearance profiles are presented in Figure 2. Considering that retinyl ester is a good marker of chylomicron core lipid, cholecalciferol seems to be removed from plasma in close association with the clearance of chylomicron lipid. However, 80 to 90% of ^{14}C-cholecalciferol was recovered in the plasma lipoprotein fraction with a density less than 1.006 g/ml as quickly as 2 min after intravenous administration, suggesting a rapid transfer of cholecalciferol from chylomicrons to other plasma components.

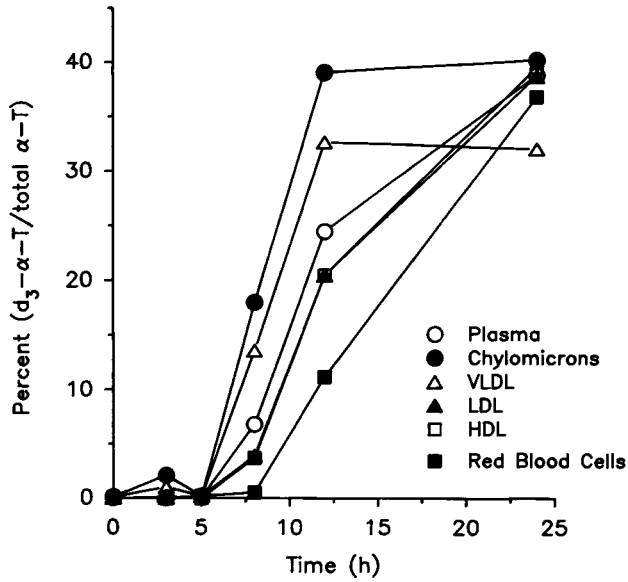

FIGURE 3. Percentages of d_3-α-tocopherol in chylomicrons, VLDL, LDL, HDL, plasma, and red blood cells (RBC) relative to the total α-tocopherol in each fraction as a function of time. These data are from a representative subject following the oral administration of 140 mg d_3-α-tocopheryl acetate. (From Traber, M. G., Ingold, K. U., Burton, G. W., and Kayden, H. J., *Lipids*, 23, 791, 1988. With permission.)

The plasma clearance of deuterium-labeled α-tocopherol in human subjects has been recently studied with the purpose of studying the time-dependent incorporation of α-tocopherol into the various plasma lipoprotein fractions.[38] The administration and subsequent measurement of deuterated α-tocopherol enables it to be distinguished from the existing levels of nondeuterated tocopherol present in human subjects. The collected blood sample was fractionated and the incorporation of deuterated α-tocopherol into various plasma lipoprotein components was assessed as a function of time. Figure 3 presents representative data from a subject who ingested d_3-α-tocopheryl acetate, and represents the time-dependent concentration profiles of d_3-α-tocopherol in the different plasma lipoprotein fractions after oral administration. The orally administered d_3-α-tocopherol first appeared in the chylomicron fraction, after which the concentrations increased in the plasma and other lipoprotein fractions. The chylomicron fraction contained the largest percentage of deuterated tocopherol (relative to nondeuterated tocopherol) at all measured time points. The next lipoprotein class to become labeled was the VLDL fraction which is consistent with the initial secretion of chylomicrons by the small intestine, uptake of chylomicron remnants by the liver, and secretion of newly synthesized VLDL containing the d_3-α-tocopherol. The subsequent metabolism of VLDL in the

systemic circulation then results in the simultaneous delivery of the d_3-α-tocopherol to both the HDL and LDL lipoprotein fractions.

B. STEROIDS

It has been suggested that the intravenous administration of lipophilic steroids could be accomplished by dissolving the drug in a lipoprotein emulsion consisting of VLDL and LDL fractions.[16] Testosterone undecanoate, a lipophilic derivative of testosterone, was administered to rats in this form without precipitation of the drug in the blood vessel or surrounding tissue. The clearance of testosterone undecanoate from the plasma was rapid ($t^1/_2$ = 6 min) which is consistent with the clearance rate of chylomicrons and VLDL from plasma. The potential application of lipoprotein-based formulations as carriers for therapeutic agents has been recently reviewed in a comprehensive manner and should be consulted for further detail.[18a]

C. DDT

The relative plasma clearance rates of chylomicron associated ^{14}C-DDT and chylomicron ^{3}H-triglyceride has been simultaneously studied by administering doubly-labeled chylomicrons to recipient rats.[14] The plasma clearance of chylomicron associated ^{14}C-DDT was very rapid ($t^1/_2$ = 2 min) and was independent of chylomicron ^{3}H-triglyceride clearance.[14] The plasma clearance profiles of the labeled DDT and triglyceride lipid are presented in Figure 4. The more rapid clearance of DDT indicated that the DDT initially associated with the chylomicron lipid was rapidly transferred to either higher density proteins or other lipoproteins after intravenous administration. These data indicate that DDT clearance from the chylomicron and metabolism of the chylomicron core triglyceride are not necessarily coupled. Subsequent studies with other hydrocarbons which are transported in the triglyceride core of chylomicrons have demonstrated that their clearance profile is largely dependent upon triglyceride lipolysis in the plasma.[46]

D. CYCLOSPORINE

Lipid preparations such as Intralipid®, a phospholipid stabilized soy bean oil emulsion for intravenous administration, can be considered as an artificial lipoprotein preparation with the principal use as parenteral lipid nutrition supplements. There has been an increasing interest in incorporating drugs into intravenous emulsion preparations, such as Intralipid®, as a means of intravenous drug administration. This approach is similar to the use of liposomes as drug carriers, although liposomes lack the core triglyceride lipid characteristics of lipoproteins.

The commercially available intravenous cyclosporine solution formulation contains Cremophore EL, a solubilizing agent which has the potential for causing numerous side-effects.[39] Venkataram and co-workers[39] attempted to

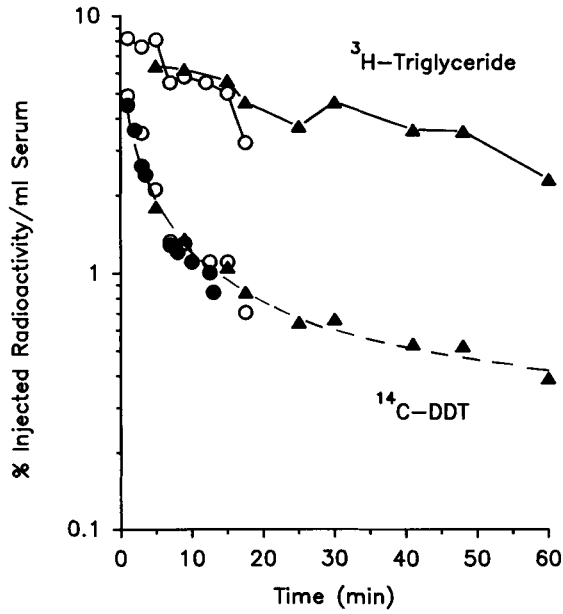

FIGURE 4. Simultaneous decay curves of chylomicron ^{14}C-DDT and ^{3}H-triglyceride in the plasma of conscious rats. The different symbols represent separate experiments in which the injected chylomicrons were either doubly or singularly labeled with either ^{14}C-DDT and/or ^{3}H-triglyceride. The broken line represents the trend of the ^{14}C-DDT decay curves. (From Pocock, D. M.-E. and Vost, A., *Lipids*, 9, 374, 1974. With permission.)

utilize cyclosporine-based liposomal and lipid emulsion formulations as suitable alternative dosage forms to the currently available solubilized formulation. They compared the pharmacokinetics of cyclosporine (CyA) in blood after intravenous administration to rabbits as either (i) liposomes, (ii) an Intralipid® emulsion, or (iii) the commercially available intravenous formulation.[39]

The terminal disposition half-life of CyA ranged between 400 and 475 min and was not significantly different between the three formulations. However, the apparent volume of distribution of CyA at steady state (Vd_{ss}) was highly dependent upon the administered formulation. The Vd_{ss} was 2.7 ± 0.2 l/kg when administered as the commercial intravenous formulation, and 7.4 ± 2.3 l/kg and 10.6 ± 2.7 l/kg when administered as the liposomal or Intralipid® formulations, respectively. These data are consistent with the clearance kinetics of endogenous lipoproteins described earlier (Section II B). Intralipid® can be reasonably considered as an artificial chylomicron since it contains triglyceride in its core, whereas the liposomes which lack core lipid may be reasonably considered as artificial HDLs. Consequently, the faster clearance of the artificial chylomicrons during the initial distributive phase might give rise to a significant increase in the apparent volume of distribution

at the steady state.[28] It is likely that the differences observed between the formulations reflect the extensive interaction between cyclosporine and the different plasma lipoproteins.

IV. LIPOPROTEIN CLEARANCE AND PHARMACOKINETICS OF THE ADMINISTERED DRUG/FORMULATION

Very few studies have assessed the effects of either endogenous or exogenous lipoprotein clearance on the pharmacokinetics of separately administered lipophilic drugs, although a number of studies have reported the effects of lipid vehicles on the lymphatic transport of orally administered drugs.[40–42] Since drugs and lipid vehicles must pass through the epithelial barrier before entering the systemic circulation, the majority of lipid vehicle effect studies have failed to separately address the effect of lipoprotein clearance from that of lipid metabolism and drug distribution. It is difficult to discriminate between the changes in the pharmacokinetic profiles of drugs which may be induced by either dietary lipids, or from changes in the metabolism of plasma lipoproteins induced by dietary lipids.

A. EFFECT OF FOOD ON SYSTEMIC LIPOPROTEIN CLEARANCE

The effect of a single meal on the kinetics of chylomicron core lipid metabolism has been investigated in rats,[30] as has the effect of different dietary patterns[27] in which rats were fed for 14 days and plasma chylomicron ^{14}C-triglyceride clearance was assessed. Section II B described the data of Redgrave and Zech[30] who studied the effect of fasting and diet on chylomicron triglyceride and cholesteryl ester clearance. As described in Table 1, the residence time of chylomicron triglycerides was 20% less after a regular diet compared to a fat-free diet, and was explained by the longer residence time of the chylomicron triglycerides after a fat-free diet due to the lower delipidation rate of chylomicrons. On the other hand, longer residence times of the chylomicron remnant and triglyceride were observed in the fasting rats, which were attributable to the altered uptake rate of remnant particles by receptors in the liver dependent on nutritional status.

Groot and co-workers[43] have studied the metabolism of triglyceride-rich plasma lipoproteins in the postprandial phase in rats which is characterized by the presence of triglyceride-rich lipoproteins of intestinal origin (chylomicrons and chylomicron remnants) in plasma. These workers found that rats fed a palm oil diet had higher serum triglyceride levels before the meal (2.0 m*M* vs. 1.1 m*M*) and reached higher levels postprandially (4.3 m*M* vs. 2.9 m*M*) when compared to rats fed a diet rich in sunflower seed oil. The relative fatty acid composition of the two lipids (palm oil vs. sunflower seed oil) are $C_{16:0}$, 45% vs. 7.2%; $C_{18:1}$, 39% vs. 19%; $C_{18:2}$, 9.8% vs. 67.5%. It was suggested that

TABLE 4
Pharmacokinetic Parameters Estimated from Plasma Cyclosporin Concentrations

Parameter	Fasting/low-fat	High-fat	Significance
CL (l/h/kg)	0.47 ± (0.10)	0.70 ± (0.16)	$P < 0.002$
MRT (hr)	2.53 ± (0.09)	2.71 ± (0.42)	NS[a]
Vd_{ss} (l/kg)	1.18 ± (0.25)	1.85 ± (0.28)	$P < 0.003$

Note: Parameters estimated (mean ± SD) following intravenous infusions (4 mg/kg) of cyclosporine to seven healthy volunteers with and without high-fat meals .

[a] Not significant ($P = 0.4$).

Taken from Gupta, S. K. and Benet, L. Z., *Pharm. Res.*, 7, 46, 1990. With permission.

sunflower seed oil triglyceride was catabolized more rapidly than palm oil triglyceride, and that furthermore, the activities of post-heparin plasma lipoprotein lipase tended to be higher in sunflower seed oil-fed animals. It was concluded that the relative hypertriglyceridemia found in palm oil-fed animals was due to less efficient catabolism and not an increased synthesis of plasma triglyceride.

B. EFFECT OF FOOD ON THE PHARMACOKINETICS OF PARENTERALLY ADMINISTERED CYCLOSPORINE

Cyclosporine (CyA) is known to be transferred from blood to lymph after intravenous injection[44] and is efficiently bound to the different classes of plasma lipoproteins. Therefore, it is reasonable to expect that lipoproteins of dietary fat origin may act as a carrier (or sink) for intravenously administered CyA. Gupta and Benet[45] determined plasma CyA concentrations after intravenous administration in healthy human subjects who had consumed either a low- or high-fat meal. Table 4 lists the mean pharmacokinetic parameters after intravenous administration of CyA and Figure 5 presents a representative plasma CyA level profile. The increased clearance (CL) and volume of distribution at steady state (Vd_{ss}) of cyclosporine observed with the high-fat meals were statistically significant, although the difference in mean residence time (MRT) was not statistically significant. The increased clearance of intravenously administered CyA following ingestion of the high-fat meal was postulated to be due to the extensive interaction of CyA with plasma lipoproteins (formed via chylomicrons due to the ingested lipid) which then facilitated the hepatic clearance of the drug. Although these studies are preliminary in nature, they indicate the potential role by which orally administered lipid can affect the pharmacokinetic profiles of lipophilic drugs via effects on the intrinsic clearance of the drug.

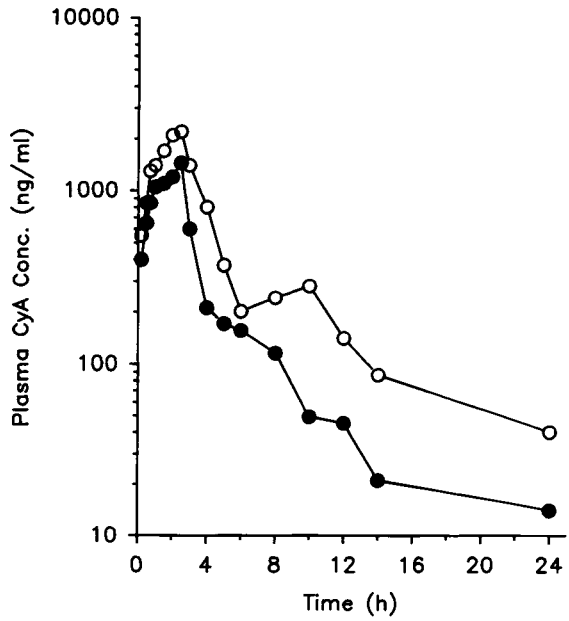

FIGURE 5. Plasma cyclosporine concentration-time profile obtained in a representative subject, following a 2.5-h intravenous infusion (4 mg/kg) of cyclosporine with (●–●) and without (○–○) high-fat meals. (From Gupta, S. K. and Benet, L. Z., *Pharm. Res.*, 7, 46, 1990. With permission.)

V. FACTORS CONTROLLING THE RELEASE OF DRUGS FROM LIPOPROTEINS

The plasma clearance of lipophilic drugs has been discussed so far in relation to that of plasma lipoproteins. In this section, the transfer of lipophilic drugs between lipoprotein fractions and from lipoproteins to plasma proteins will be discussed. The means by which drug transfer occurs and the factors which control the transfer will also be addressed.

The binding ability of lipophilic substances to human serum lipoproteins (VLDL and LDL) has been demonstrated to correlate reasonably well with their lipophilic character as estimated by measures such as retention time in reverse phase HPLC.[16] Consequently, a number of studies have been conducted to determine whether the distribution of lipophilic compounds between plasma lipoproteins is correlated with their inherent lipophilicity. The chlorinated hydrocarbons and lipophilic vitamins have been most extensively studied in this regard.

A. CHLORINATED HYDROCARBONS

DDT and other chlorinated hydrocarbons have been extensively studied as model compounds for lymphatic transport because of their very high lipophilicity

and resistance to metabolism during intestinal uptake. The disappearance of DDT associated with chylomicrons from the systemic circulation has been shown to be rapid and partly independent of the presence of the liver and of triglyceride hydrolysis, with some DDT being transferred from chylomicrons to albumin or other plasma proteins before tissue uptake.[14] However, among the different chlorinated hydrocarbons, this observation appears to be a specific characteristic of DDT.

Vost and MacLean[46] performed a comparative study on the plasma clearance of four highly lipophilic aryl and alkyl hydrocarbons (benzo(a)pyrene, DDT, hexadecane, and octadecane) which were selected to represent a graded range of polarity. Hexadecane, octadecane, and benzo(a)pyrene were cleared from the plasma at the same rate as chylomicron triglycerides, which is consistent with lipoprotein lipase-dependent hydrocarbon clearance. However, DDT was cleared more rapidly than these other three hydrocarbons (Section III C). Despite these differences in the rate of clearance between DDT and the other hydrocarbons, the major plasma acceptor for each of the hydrocarbons was the HDL.

Skalsky and co-workers[47] discussed the binding of organochlorine pesticides to plasma proteins and their role in systemic distribution. The majority of the organochlorine pesticides bind largely to LDL as well as VLDL, although chlordecone appears to be primarily associated with the HDL fraction. Busbee and co-workers suggested that benzo(a)pyrene was taken up by LDL more efficiently than by VLDL and HDL.[12]

These fragmented observations suggest that, in general, chlorinated hydrocarbons transfer rapidly from chylomicrons to HDL and the other higher density plasma proteins in the blood. This is possibly due to a greater structural assimilation with surface proteins rather than with the neutral lipid core of lower density lipoproteins, although further studies are necessary to clarify the specific aspects of the interaction and transfer process.

B. LIPOPHILIC VITAMINS

Studies of the plasma distribution of β-carotene in humans after oral administration revealed that peak carotenoid increments in chylomicron and VLDL fractions occurred much earlier than those in the LDL fraction.[48] Several observations have suggested that rapid equilibration and exchange of β-carotene between plasma lipoprotein fractions does not occur.

The absorption of retinol and cholecalciferol has been extensively studied. Retinol, in the form of retinyl esters, enters the lymphatic pathway in association with chylomicrons and remains associated with the core lipid of chylomicrons and their remnants. In contrast, cholecalciferol is transferred from chylomicrons to other plasma fractions with only a fraction of the cholecalciferol following the chylomicron remnants in their uptake by the hepatocytes.[37]

Early questions on how and where α-tocopherol was incorporated into human lipoproteins were due to the lack of an adequate metabolic tracer that

could be used for human studies. Traber and co-workers[38] solved this problem by utilizing deuterium-substituted α-tocopherol. From these studies (Section III A), the time course of appearance of deuterated α-tocopherol increased most rapidly in chylomicrons, then in VLDL, followed by essentially identical increases in LDL and HDL, and finally appearing in red blood cells. This pattern of appearance of deuterated α-tocopherol is consistent with the following concept which is also applicable to retinyl esters. Newly absorbed α-tocopherol is secreted by the intestine in association with chylomicrons which drain into the systemic circulation. Subsequently, chylomicron remnants are taken up by the liver from which the α-tocopherol is secreted in VLDL which are metabolized in the circulation, resulting in the simultaneous delivery of α-tocopherol to the LDL and HDL fractions. The transfer of α-tocopherol between plasma lipoproteins was recently studied by Granot and co-workers[49] who found that the external addition of a neutral lipid transfer protein did not affect the transfer process.

Based on the limited discussions above, the release of highly lipophilic drugs from chylomicrons (or chylomicron-like emulsions) after intravenous administration may be hypothesized to include the following: for low log PC (partition coefficient) compounds, diffusion is probably dominant, and consequently drug release is almost independent of chylomicron metabolism. In contrast, for high log PC compounds such as the lipophilic hydrocarbons and drugs such as testosterone undecanoate, the metabolism of triglyceride lipid may play a critical role. In this case, where the highly lipophilic drug is associated with the chylomicron lipid, lipoprotein lipase-based lipolysis of the core triglyceride first occurs followed by concomitant release of drug. Subsequently, diffusion or transfer of drug to other lipoproteins may occur. In this case, protein binding is another important factor which may affect drug release and distribution. Compounds having high protein/lipoprotein binding affinity, such as DDT, may exhibit a more rapid plasma clearance than chylomicron triglyceride.

VI. SUMMARY

The fate of lipoproteins in the systemic circulation has been briefly reviewed, and with the aid of pharmacokinetic models, the contribution of various tissues to lipoprotein clearance was clarified in a semi-quantitative manner. Chylomicrons appear to be converted to chylomicron remnants by lipoprotein lipase on extrahepatic capillary beds which are taken up by the liver and subsequently secreted as newly synthesized VLDL. The metabolism of VLDL in the circulation results in the production of LDL and HDL.

Most available data suggest that the clearance of lipoproteins may affect the clearance of lipophilic drugs from the systemic circulation. Retinyl esters and α-tocopheryl esters associated with the core lipid of chylomicrons are cleared from the circulation in concert with chylomicron metabolism. On the

other hand, chlorinated hydrocarbons and cholecalciferol are associated with plasma proteins as well as with chylomicrons in the systemic circulation. Consequently, they are removed from chylomicrons more rapidly in the systemic circulation than the metabolism of the chylomicron.

The clearance of lipoproteins appears to be affected by concomitant drug administration and various disease states. This observation may prove to be an important consideration, particularly for the systemic delivery and delivery of lipophilic drugs. Further studies are required to understand the clearance and distribution of drugs that associate with chylomicrons and other lipoproteins. Perhaps novel pharmacokinetic models need to be developed to quantitatively assess this interesting phenomena.

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Chapter 7

LYMPHATIC TRANSPORT OF POLAR DRUGS AFTER GASTROINTESTINAL ADMINISTRATION

Hiroshi Yoshikawa and Shozo Muranishi

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I. INTRODUCTION

After absorption through the mucosa of the gastrointestinal tract, drug molecules may be transported to the systemic circulation via either the portal blood or intestinal lymphatics. While it is well known that the lymphatics of the small intestine play a special part in the transport of dietary lipids and lipophilic compounds such as cholesterol,[1,2] the lipid soluble vitamins,[3–5] Sudan Blue,[6] and naftifine,[7] details regarding the transport and absorption of polar drugs via the intestinal lymphatics after gastrointestinal dosing have not been clear. As discussed in Chapter 4, the main route for the absorption of orally administered polar drugs is via the portal blood due primarily to the much greater flow than occurs with the intestinal lymphatics. However, there is much interest in enhancing the lymphatic delivery of some polar drugs for (i) the treatment and prevention of lymphatic metastases by anticancer agents, (ii) the potential enhancement of immunological reactions by biological response modifiers, and (iii) the avoidance of the hepatic first pass effect.

Some attempts have been made to enhance the lymphatic delivery of polar compounds through the application of lipophilic prodrugs;[8] however, it appears that the design of lymphotropic and active compounds for hydrophilic drugs is a complex task. The success of different strategies for targeting drugs to specific sites within the body is often dependent on the anatomical characteristics of the transport barriers. This realization has special significance in terms of lymphatic transport as there are some major anatomical differences between blood and lymphatic capillaries of the intestine (see Chapter 1 for details). For example, there are gaps between the intercellular junctions of the endothelial cells lining the lymphatic capillaries, which is not the case with the blood capillaries, and the basement membrane is essentially continuous within the blood capillary endothelium whereas it is mostly absent within the lymphatic vessels. These features are responsible for the lymphatic transport of macromolecules, microparticles, and lipoproteins. These molecules are able to pass through the relatively "open" structure of the lymphatic endothelium and are excluded by the relative "integrity" of the blood capillaries. The uptake of microparticulates by the gastrointestinal tract is addressed in Chapter 9.

In this chapter, we describe the lymphatic transport of some different large molecular weight polar compounds after gastrointestinal dosing and the requirement for the coadministration of absorption enhancers to facilitate permeation through the mucosal barrier. Specific examples are presented of our own work where we have attempted to potentiate the selective lymphatic delivery of anticancer drugs for the treatment of lymphatic metastases of gastrointestinal cancer.

II. THE INTESTINAL BLOOD-LYMPH BARRIER AFTER ADMINISTRATION OF POLAR COMPOUNDS

A. SMALL MOLECULAR WEIGHT COMPOUNDS

There is a reasonable amount of data concerning the absorption and lymphatic transport of small molecular weight lipophilic substances (see Chap-

ters 3 and 4), and this has enabled identification of the necessary prerequisites for transport. In contrast, there are limited studies in which lymphatic transport of polar compounds has been evaluated. DeMarco and Levine have studied the lymphatic transport of several polar drugs in the rat (e.g., *p*-amino salicylic acid, sulfonamides and tetracycline) after duodenal administration.[9] They found that the concentration of these polar drugs in the blood and lymph were similar, although the extent of absorption via the lymphatics was less than 1.0% of the administered dose due to the much lower flow rate. Similar results have been reported for the small molecular anticancer agent 5-fluorouracil after administration with or without lipid-surfactant mixed micelles, which are utilized as absorption promoters.[10] Sieber and co-workers have demonstrated the lymphatic transport of various foreign and natural chemicals in the rat after duodenal administration.[11] In their studies, the lymphatic transport of hydrophilic compounds such as salicylic acid, isoniazid and hexanoic acid was very small, whereas significant quantities of some lipophilic compounds (octadecanol and *p,p'*-DDT) were transported in the chylomicron fraction of intestinal lymph. These data demonstrate that the gastrointestinal lymphatics generally do not play a major role in the transport of small molecular weight polar drugs.

B. MACROMOLECULES

1. Gastrointestinal Absorption of Macromolecules via the Lymph

The relative permeability of the blood and lymphatic capillaries to large water-soluble molecules is often studied using the capillary perfusion method.[12–14] Mayerson and co-workers have studied the relative permeabilities of different blood capillaries to macromolecules and demonstrated that although the enteral blood capillary is less permeable than the hepatic capillary, it is much more permeable than the cervical capillary due to the presence of fenestrae within the endothelial cell structure.[12] Alexander and co-workers proposed in 1936 that orally administered egg white protein in the canine could gain access to the systemic circulation via the intestinal lymphatics.[15] More recent studies have demonstrated that several types of botulinum toxin (900 kD) and enteric endotoxin can appear in high titres in thoracic duct lymph after oral administration,[16–18] indicating that the intestinal lymphatics may play a role in the transport of these macromolecular toxins. The lymphatic transport of horseradish peroxidase (HRP, 40 kD) has been studied in a rat jejunum preparation and levels of HRP were apparent in both portal venous blood and mesenteric lymph.[19] In a subsequent study, the absorption of bovine serum albumin from the rat duodenum was studied and during the 3-hr period after administration approximately 0.8% and 1.1% of the administered dose appeared in the intestinal lymph and portal vein, respectively.[20] The absorption of elastase (24 kD) has been studied in the rat where it was demonstrated that elastase levels in the thoracic duct lymph were approximately 10 times higher than peripheral blood levels.[21] From these studies it was calculated that approximately two thirds and one third of the absorbed elastase (corresponding to approximately 0.05 to 0.15% of the dose) was absorbed via the portal blood and lymph, respectively. There are very few studies which have systematically

TABLE 1
Classes of Absorption Enhancers Effective After Gastrointestinal Administration

Class	Enhancing effectiveness	Remark on absorption barrier and side effect	Examples
I	Strong and fast reactivity, with fast recovery of barrier function	Comparatively safe	Fatty acids such as capric, oleic, linoleic, and arachidonic acid, and their monoglycerides Acylcarnitines; alkylsaccharides such as LM and OG; azone
II	Moderate and fast reactivity, with fast recovery	Comparatively safe	Bile salts: cholate and STDHF Salicylates: salicylate, 3-methoxysalicylate, and 5-methoxysalicylate Homovanilate
III	Strong or moderate reactivity, with slow recovery	Cell or tissue disturbance possible	Strong surfactants such as SLS, polyoxy-23 lauryl ether, BL 9-EX, Brij 35, etc. Chelating agents such as EDTA, EGTA, citric acid, phytic acid, enamine derivatives, and DEEMM
IV	Moderate reactivity as water miscible solvents	Comparatively safe, but cautious of systemic side effect	DMSO, DMAC, DMF, NMP, and ethanol

From Muranishi, S., *Crit. Rev. Ther. Drug Carrier Syst.*, 7, 1, 1990. With permission.

examined the absorption of macromolecules via the lymph and portal blood, which is probably due to the problems associated with their detection and inherently poor permeability through the enteral mucosa. An excellent and comprehensive review has recently been published on the absorption of peptides and proteins by the gastrointestinal lymphatics.[21a]

2. Enhancement of Gastrointestinal Absorption

In recent years a range of compounds with widely varying structures have been identified as effective absorption enhancers for compounds after gastrointestinal administration. Muranishi has classified these different agents in terms of their enhancing ability and their side effect profile on the gastrointestinal mucosal barrier, and this classification is presented in Table 1.[22] Naturally occurring polar lipids such as saturated medium chain fatty acids, long chain unsaturated fatty acids and their monoglycerides are classified as class I promoters which are the most effective and are comparatively safe. Also included in this classification are synthetic enhancers with Azone (1-dodecylazacycloheptan-2-one)[23] and n-lauryl-D-maltoside (LM),[22] being two

well-studied examples. The majority of these compounds are poorly water soluble and are therefore administered in either co-solvent or solubilized systems. The class II enhancers are less effective than the class I enhancers, and representative examples are bile salts and their derivatives,[24,25] and the salicylates.[26] Class III enhancers are relatively effective at promoting the absorption of compounds although the rate of tissue recovery is slow and tissue damage is often observed. Strong surfactants such as sodium lauryl sulfate (SLS) and a number of different chelating agents fall into this category.[27,28] The utility of some enamine derivatives and the diethylethoxymethylenemalonates (DEEMM) are believed to act as calcium ion chelating agents.[29] From a structural standpoint, the compounds exhibiting the highest degree of enhancing activity generally contain these characteristics: (i) a lipophilic group such as a C_{10}-C_{20} hydrocarbon chain or steroid ring, and (ii) hydrophilic moieties such as carboxyl, sulfate, glyceryl hydroxy group, and mono- or disaccharides units.[22] Of these absorption enhancers, we have found that the enteral mucous membrane can be made reversibly and safely permeable to poorly absorbable drugs with the aid of lipid (unsaturated fatty acids or their long chain monoglycerides) surfactant mixed micellar (MM) formulations.[10,22,30–33] In microscopic histological studies, treatment of the rat rectal lumen with micellar oleic acid resulted in the loss of the glycocalyx, although the structure of the epithelial cells remained intact.[22,34]

The effect of four different enhancers on the absorption of 6-carboxyfluorescein (CF, molecular weight 376) has been studied by pretreating the rat large intestine with the different agents, and the resulting plasma profiles are presented in Figure 1. CF is a model polar compound which is poorly absorbed from the gastrointestinal tract. The enhancers studied were SLS, oleic acid-surfactant (hydrogenated castor oil, HCO-60) mixed micelles, EGTA (ethylene glycol-bis(β-aminoethyl ether), N,N,N′,N′-tetraacetic acid which is a mild chelating agent), and LM. The increased plasma levels of CF were due to enhanced absorption due to the pretreatment of the intestine with the different enhancer systems. Importantly, when the intestine was washed to remove the enhancers after the initial experiment and free CF was administered, the previous marked enhancement observed with the oleic acid and LM system was no longer apparent, indicating the apparent reversibility of the enhancement. Muranushi et al. indicated that exposure of the intestine to micellar fatty acids acts to both increase the influx and efflux of poorly permeable drugs and that these effects can be reversibly diminished by lumenal washing.[33] These data indicate that the observed increase in intestinal permeability occurred without apparent irritation or injury to the intestinal epithelial cells. Subsequent studies employing electron spin resonance (ESR) and nuclear magnetic resonance spectroscopy (^{1}H-NMR) by Muranishi and co-workers using model liposomal membranes have suggested that the enhanced permeability of the intestinal epithelium caused by the fatty acid micellar systems is associated with induced disorder in the interior hydrophobic regions of the

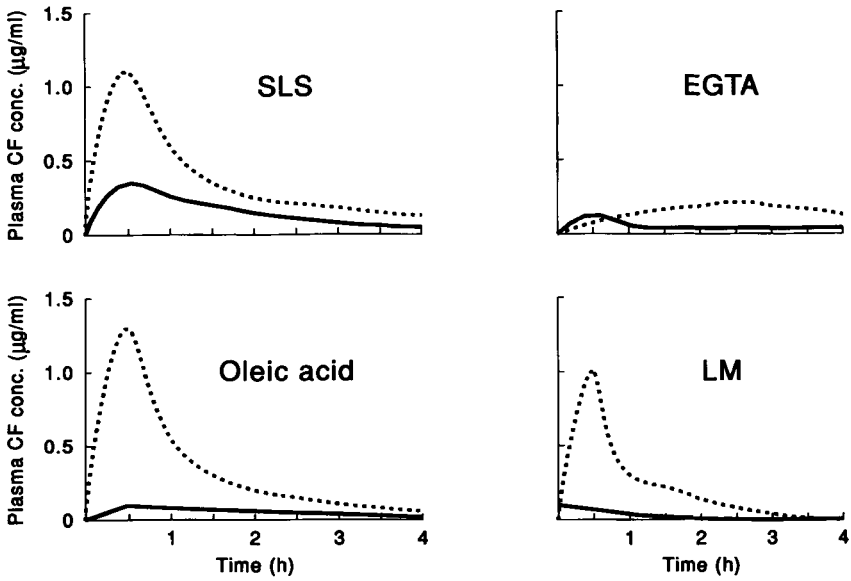


FIGURE 1. Plasma profiles of 6-carboxyfluorescein (CF) after administration to the large intestine of the rat. The dashed lines depict plasma CF levels after pretreatment with either with SLS solution, Oleic Acid-HCO-60 solution, EGTA solution, or LM solution; the solid lines represent the levels of CF after the subsequent administration of CF without an enhancer. (From Muranishi, S., *Crit. Rev. Ther. Drug Carrier Syst.*, 7, 1, 1990. With permission.)

membrane and interactions with the polar head groups of the membrane phospholipids.[35]

It is generally accepted that both the transcellular (through cell membranes) and paracellular routes (through tight junctions) offer significant resistance to the transport of polar and macromolecular substances. In terms of the enhanced absorption of macromolecules from the rat intestine induced by the mixed micellar systems, we found that the increased role of the paracellular route was dependent on the molecular size of administered macromolecules.[36]

Proteins are another important constituent of cell membranes and their diverse functions range from effects on membrane permeability to playing a role in cellular communications. Therefore, the effect of the MM systems on membrane proteins is an interesting matter. The sulphydryl (SH) groups present in biomembrane proteins, and their involvement in the tight junctions of the epithelial barrier, play an important role in the transport process. Murakami et al. investigated the contribution of membrane-bound proteins to the enhanced permeability observed with MM systems and several SH-modifying reagents.[37] In these studies, pretreatment of the intestinal mucosa with permeable SH blockers reduced the enhancing activity of oleic acid whereas impermeable SH blockers had no effect on the enhanced permeation associated with oleic acid. Muranishi has confirmed that pretreatment of the intestinal mucosa with permeable SH blockers decreases the enhanced permeation of macromolecules

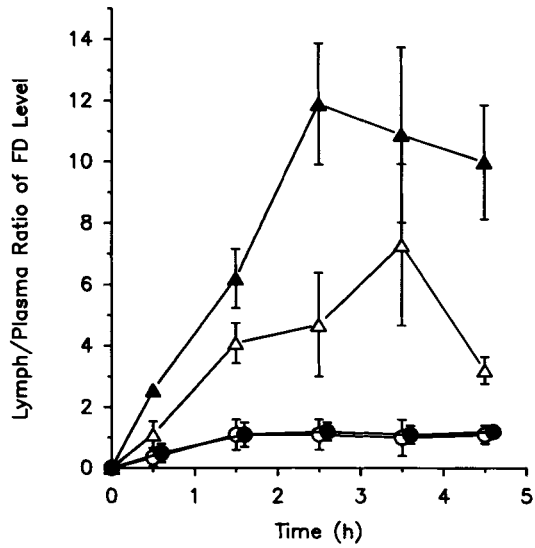


FIGURE 2. Ratios of the thoracic lymph concentration/plasma concentration of different molecular weight fluorescein-labeled dextrans (FD) after administration into the lumen of the *small intestine* of the rat. The approximate molecular weights were 10 kD (FD-10, ○–○); 20 kD (FD-20, ●–●); 40 kD (FD-40, △–△); 70 kD (FD-70, ▲–▲). Each value represents the Mean ± S.E., n = 4 – 5 (From Yoshikawa, H., Takada, K., and Muranishi, S., *J. Pharmacobio-Dyn.*, 7, 1, 1984. With permission.)

which are primarily absorbed through the paracellular route due to the effects of the MM systems.[38] In addition to effects on the paracellular route, data from these studies also demonstrated an inhibitory effect of the SH reagents on the transcellular route of absorption. Therefore, interaction of lipids such as oleic acid with key SH proteins in both the paracellular and transcellular routes is considered to contribute to the enhanced permeability of intestinal mucosa caused by MM. From the results and data presented in the cited reports, the use of MM is considered to be a feasible means to assess the effective selective permeability of poorly absorbable macromolecules for either the blood or lymph capillaries.

3. Lymphotropic Characteristics and the Effect of Molecular Weight

Figure 2 presents the ratio of the concentrations in thoracic duct lymph and peripheral plasma (L/P) of different molecular weight fluorescein-labeled dextrans after administration with a linoleic acid MM system into the lumen of the small intestine of the rat.[39] The approximate molecular weights of the administered fluorescein-labeled dextrans were 10, 20, 40, and 70 kD and they are abbreviated as FD-10, FD-20, FD-40, and FD-70, respectively. Over the 5-hr time course of the experiment, the L/P ratios increased with increasing molecu-

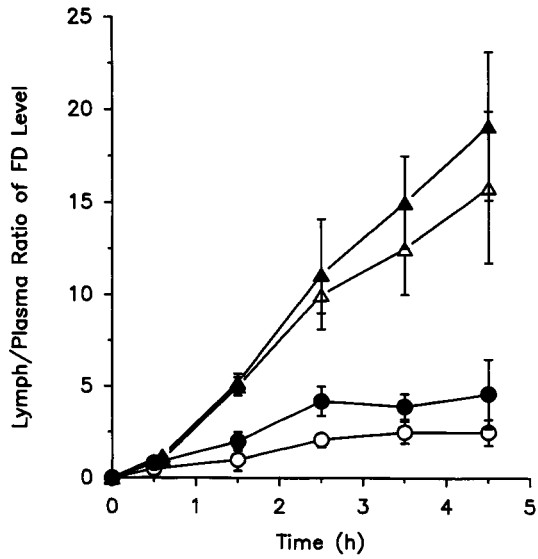


FIGURE 3. Ratios of the thoracic lymph concentration/plasma concentration of different molecular weight fluorescein-labeled dextrans (FD) after administration into the lumen of the *large intestine* of the rat. The approximate molecular weights were 10 kD (FD-10, ○–○); 20 kD (FD-20, ●–●); 40 kD (FD-40, △–△); 70 kD (FD-70, ▲–▲). Each value represents the Mean ± S.E., n = 4 – 6 (From Muranishi, S., *Rectal Therapy*, Prous Publishers, Barcelona, 1984. With permission.)

lar weight. The L/P ratio was approximately unity for the FD-10 and FD-20 molecules; however, it increased to between 1 and 7 for FD-40, and to between 3 and 12 for FD-70. Although the plasma and lymph concentrations of FD-10 and FD-20 were similar, the increased molecular weight of the FD-40 and FD-70 conjugates were associated with significantly higher levels in the lymph than in the plasma. These data suggest that the selective permeability of FD from the rat small intestine (employing the MM enhancers) for the lymphatic vessels, relative to the blood capillary, occurred with an increase in molecular weight from between 20 and 40 kD which corresponds to an approximate molecular diameter of 64 to 88 Å.

The relative blood-lymph permselectivity of the same fluorescein conjugated dextrans after administration with MM to the rat large intestine is shown in Figure 3.[40] In the large intestine, lymph levels of FDs of greater than 10 kD (approximate diameter 46 Å) were higher than the peripheral blood levels, with the L/P ratios of FD-40 and FD-70 approaching values of approximately 20 to 25. These data demonstrate the apparent differences in the threshold molecular weight and the increased L/P ratio after administration in either the small or large intestine. In terms of selectivity, the concentration of the different FD conjugates in lymph from either the small or large

intestine was largely independent of molecular weight, whereas the plasma levels of the FD conjugates decreased with increasing molecular weight (data not shown).[39,40] Therefore, the differences in the L/P ratios appear to be a manifestation of the molecular weight dependency of access to the blood capillaries. Richardson and co-workers have reported that the permeability of the colonic blood capillaries is somewhat different from those present in the ileum.[41]

4. Effect of Molecular Species

The effect of molecular species on the relative selectivity for either the blood or lymph capillaries has been investigated with a number of different macromolecules with similar molecular weight yet different molecular structure. In this regard, we have studied the lymphatic absorption of interferon (IFN) with the aid of MM from the rat large intestine,[32,34,39,40] as the large intestine is more sensitive to the enhancing properties of the MM than the small intestine. The antitumor activity of IFN has been reported to be independent of IFN concentrations in peripheral blood and most closely associated with the localized IFN concentrations present within the region of the tumor.[42] Furthermore, the lymphatic concentrations of IFN are an important therapeutic endpoint of the likely immunomodulation activity due to the greater density of lymphoid cells present within the lymphatics rather than the blood.[43] When human fibroblast IFN (HuIFN-β, glycosylated 20 kD protein) was administered to the lumen of the large intestine in the rat (dose: 3×10^6 IU/rat administered as a saline solution) there was no detectable HuIFN-β present in either the peripheral blood or thoracic duct lymph (minimum detectable levels in either blood or lymph was 30 to 45 IU/ml).[44] Figure 4 presents the concentration profile of HuIFN-β in lymph and peripheral serum after administration of the same dose (3×10^6 IU/rat) with linoleic acid-HCO-60 mixed micelles (MM) to the rat large intestine. The coadministered MM produced high concentrations of HuIFN-β (540 to 1630 IU/ml) in the thoracic duct lymph over the 5-hr post-dosing period with very little HuIFN-β appearing in the peripheral blood.[44] These data indicate that the HuIFN-β, when administered with the MM formulation, gained access to the lymphatics more rapidly than the blood and with extremely high selectivity, as the calculated L/P HuIFN-β ratios in Figure 4 ranged between 17 and 48. The absorption of HuIFN-α (18 kD, nonglycosylated protein) from the large intestine of the rat was studied using the same methodology, and with the aid of MM formulations, the smaller molecular weight HuIFN-α was also selectively delivered into the lymphatics, as the L/P ratios were approximately 10 to 20 over the 5-hr period post dosing (Figure 5).[45] These data demonstrate that the lymphotropic characteristics of HuIFN, when absorbed from the rat large intestine due to the co-administered MM, is essentially independent of the molecular form of HuIFN (i.e., α or β). Bocci and co-workers have studied the absorption of human lymphoblastoid IFN from the rectum of the rat when coadministered with ursodeoxycholate as a promoting adjuvant.[46] It was determined that a significant contribution to the

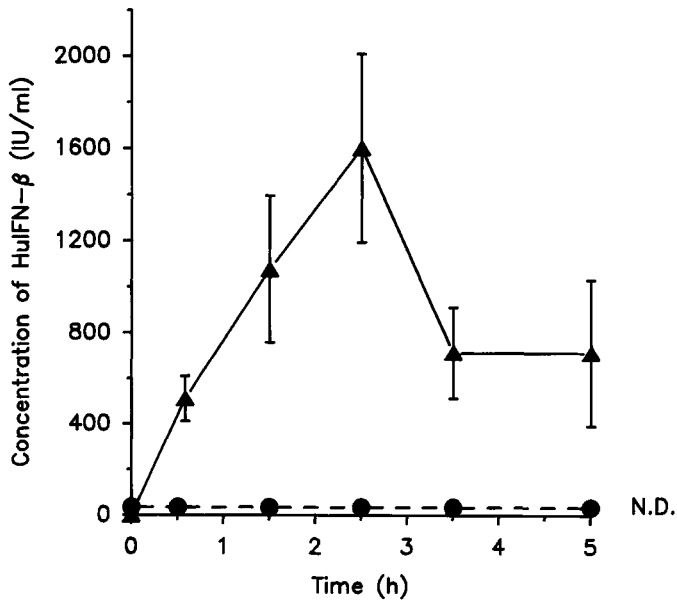


FIGURE 4. Serum (●–●) and lymph (▲–▲) concentrations of HuIFN-β after administration as a linoleic acid-HCO-60 mixed micellar system (MM) into the lumen of large intestine of the rat. The minimum detectable levels of HuIFN-β in the serum were 30 – 45 IU/ml (N.D. = not detectable). Data are presented as Mean ± S.E., n = 5 (From Yoshikawa, H., Takada, K., Muranishi, S., Satoh, Y., and Naruse, N., *J. Pharmacobio-Dyn.*, 7, 59, 1984. With permission.)

colo-rectal absorption of the compound was due to the lymphatics draining the area of administration.[46]

When HuIFN has been formulated as a suppository for rectal administration, similar requirements in terms of absorption enhancement as seen with the small and large intestine of the rat were observed.[47] Hydrophobic suppository bases containing either the lipid alone (linoleic acid) or the surfactant (HCO-60) alone failed to enhance the rectal absorption of HuIFN-β. However, suppository bases which contained both lipid and surfactant significantly facilitated the rectal absorption of HuIFN-β with selective delivery via the lymph (Figure 6). The maximum thoracic lymph concentration and the extent of HuIFN-β absorption via the thoracic lymph after rectal administration as a suppository was approximately 20% of that observed when administered as a MM-based solution to the intestines. The rate of diffusion of HuIFN-β and the MM components in the molten suppository is much lower than in the MM solution, and this may be a major factor which limits the rectal absorption of HuIFN.

The extent of lymphatic absorption of water-soluble macromolecules administered into the lumen of the gastrointestinal tract appears to be primarily dependent on a molecular sieving mechanism owing to the limited permeability of the blood capillary wall. As demonstrated in Figure 3, administration of

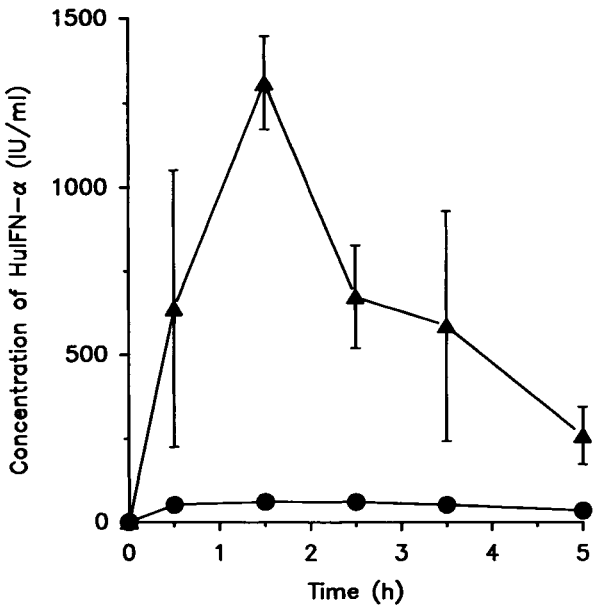


FIGURE 5. Serum (●–●) and lymph (▲–▲) concentrations of HuIFN-α after administration as a linoleic acid-HCO-60 mixed micellar system into the lumen of the large intestine of the rat. The serum levels were at or below the sensitivity of the assay. Data are presented as Mean ± S.E., n = 3 (From Yoshikawa, H., Takada, K., Satoh, Y., Naruse, N., and Muranishi, S., *Pharm. Res.*, 2, 249, 1985. With permission.)

a 20 kD fluorescein-dextran conjugate (FD-20) with MM in the large intestine of the rat produced a L/P ratio of 5, whereas the L/P ratio for HuIFN-β when administered with MM was between 17 and 48 despite a similar molecular weight. In terms of these different L/P ratios for similar molecular weight species, Bohrer et al. reported that the molecular species (i.e., molecular shape or configuration) can influence the permeability of the glomerular capillary wall.[48] The fenestrae present in the glomerular capillary wall is similar to that present within the enteral blood capillary endothelium, so it is possible that there may be a specificity in terms of molecular species. Therefore, the different chemical forms of dextran (polysaccharide) and the different HuIFNs (protein or glycoprotein) may have determined the differing lymphatic transport profiles observed in these studies.

III. SELECTIVE LYMPHATIC DELIVERY OF ANTICANCER DRUGS

A. LYMPHOTROPIC DRUG DELIVERY AFTER GASTROINTESTINAL ADMINISTRATION

The selective affinity and delivery of drugs for the treatment of a particular disease state is a desirable endpoint, and this goal has special significance for

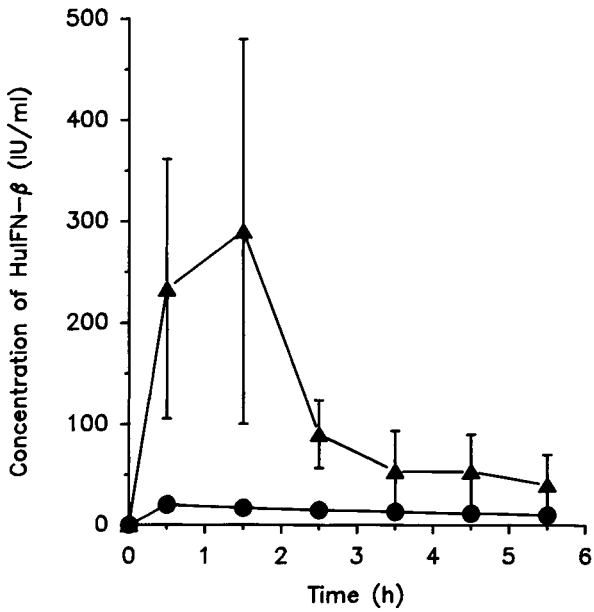
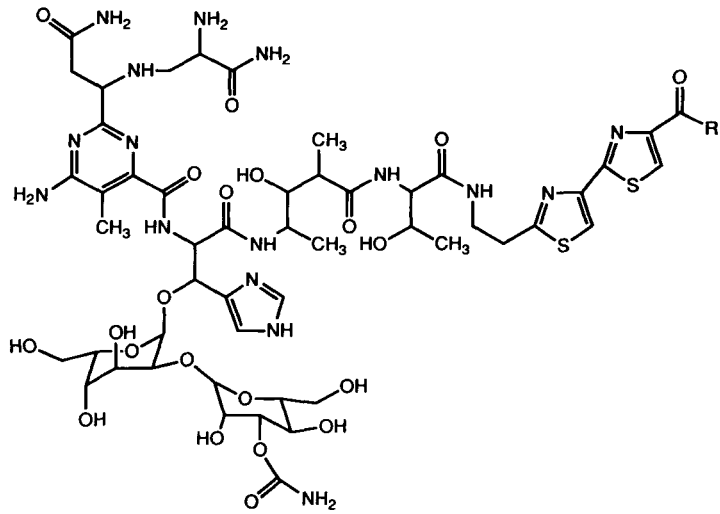
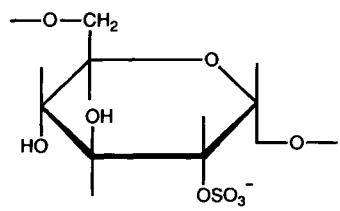


FIGURE 6. Serum (●–●) and lymph (▲–▲) concentrations of HuIFN-β in rats after rectal administration of suppositories containing linoleic acid and HCO-60. Data are presented as Mean ± S.E., n = 4. (From Yoshikawa, H., Takada, K., Satoh, Y., Naruse, H., and Muranishi, S., *Pharm. Res.*, 3, 116, 1986. With permission.)

the treatment of lymphatic metastases. After parenteral administration, a variety of delivery systems for some anticancer drugs such as emulsions and particulate systems can potentiate the tumor metastasis treatment due to selective delivery via the lymphatic route (see Chapters 8 and 9 for details). Although the oral and rectal routes of administration are useful and convenient, there are many anticancer agents which are ineffective when administered by these routes. In addition to the potential first pass effect of orally administered anticancer drugs, the polar nature and large molecular weight of many of the different agents contribute to their relatively poor absorption. The promotion of lymphatic drug absorption will avoid the hepatic first pass effect, and therefore the lymphatics could be considered an important target site in chemotherapy of gastrointestinal cancer, as well as a means to increase the bioavailability of such drugs after gastrointestinal dosing. When designing either oral or enteral anticancer drug delivery systems to treat lymphatic metastasis of gastrointestinal cancer, the following are some points to be considered: (i) binding of the drug to an appropriate lymphotropic carrier, (ii) transfer of the drug-carrier complex through the enteral barrier, and (iii) maintenance of antitumor activity in either the complex form or after dissociation and subsequent release of free drug in the lymphatics.



BLEOMYCIN



DEXTRAN SULFATE

FIGURE 7. Chemical structure of bleomycin (BLM, where R = terminal amines) and a partial structure of dextran sulfate (DS).

Based on these requirements and the results described in Section I, we have designed and evaluated a new lymphotropic delivery system for anticancer drugs after gastrointestinal administration. Initially, bleomycin (BLM, approximate molecular weight 1500, Figure 7) was chosen as a drug candidate as it is a water-soluble, basic glycoprotein which is poorly absorbed after gastrointestinal administration. A macromolecular complex[50] was formed between cationic BLM and anionic dextran sulfate (DS, approximate molecular weight 500 kD, Figure 7). The DS acted as a lymphotropic carrier and the previously described MM system was used as the absorption enhancer to facilitate penetration of the large complex through the enteral mucosa (Table 2). The

TABLE 2
Lymphotropic Drug Delivery Systems for the Enhancement of Anticancer Drug Absorption after Gastrointestinal Dosing

Drug	Lymphotropic carrier	Molecular weight of carrier	Type of complex	Absorption enhancer
Bleomycin (BLM)	Dextran sulfate (DS)	Approximately 500 kD	Ion-pair	Lipid-surfactant mixed micelles (MM)
1-hexyl-carbamoyl-5-fluorouracil (HCFU)	β-cyclodextrin (polyβCD)	Approximately 10 kD	Hydrophobic inclusion	Lipid-surfactant mixed micelles (MM)

concept of this delivery system is depicted in Figure 8 — (a) neither free BLM nor the BLM-DS complex (BLM-Y) can normally penetrate the enteral barrier; (b) the co-administration of an absorption enhancer (X) such as a MM system enables free BLM to permeate the intestinal mucosa with BLM subsequently being delivered into the blood and lymph without inherent selectivity; and (c) when BLM-Y is co-administered with X, the macromolecular complex is transported through the mucosa due to X and is selectively transferred to the lymphatics due to the size of the BLM-Y complex.[51]

Figure 9 presents the concentration of BLM in thoracic lymph or plasma after administration of either free BLM or the BLM-DS complex to the large intestine of the rat. These studies were extended to include evaluation of the effect of the MM enhancer system. There were very low levels of BLM in the plasma and lymph when administered as free BLM, although both these levels increased approximately tenfold when MM were co-administered. The levels of BLM after administration of BLM-DS without the MM enhancer were similar after administration of the free BLM. Importantly, when the BLM-DS complex was administered with the MM system, the lymph levels increased by a 15- to 20-fold factor while the plasma levels only increased by a factor of about 3 to 5.[51] Furthermore, in terms of the cumulative amount of BLM transported in the lymph over the 5-hr period post dosing, the BLM-DS complex when administered with the MM enhancer was most effective.[51]

The stability of the BLM-DS complex in the enteral lumen and the tissue is an important factor affecting the lymphotropic transport and selectivity of BLM absorption. By using gel filtration (size exclusion) chromatography, it was determined that almost all of the BLM-DS complex was intact within the large intestinal lumen of the rat, with about 50% of the complex being dissociated within the large intestinal tissue, and over 95% of the absorbed BLM being detected as free BLM in the lymph and blood.[52] The dissociation profile represented by these data is reasonable since BLM-DS is an ionic complex, and

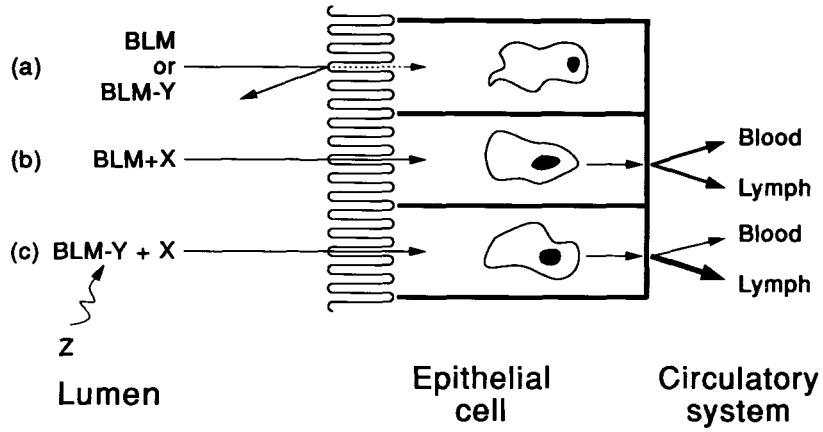


FIGURE 8. Proposed scheme for the enhanced absorption and lymphatic transport of BLM when complexed with a high molecular weight agent such as dextran sulfate (DS). X is an absorption inducer such as monoolein-sodium taurocholate mixed micelles; Y is a high molecular weight complexing agent such as DS; and Z is an intestinal labilizer which may dissociate the ionic complex. (From Yoshikawa, H., Muranishi, S., Kato, C., and Sezaki, H., *Int. J. Pharmaceut.*, 8, 291, 1981. With permission.)

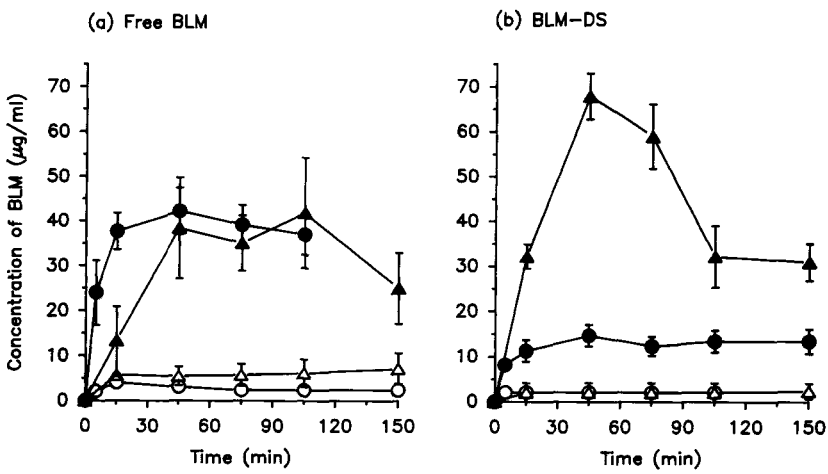


FIGURE 9. Concentration of BLM in plasma (○ or ●) and thoracic duct lymph (△ or ▲) after administration into the large intestine of the rat. (a) administration of free BLM; (b) administration of BLM-DS complex. The open symbols represent BLM concentration without co-administered mixed micelles, and closed symbols represent BLM concentration when administered as a mixed micellar solution (monoolein-sodium taurocholate). Data are presented as Mean ± S.E., n = 4 – 8. (From Yoshikawa, H., Muranishi, S., Kato, C., and Sezaki, H., *Int. J. Pharmaceut.*, 8, 291, 1981. With permission.)

TABLE 3
Metastatic Lymph Node Weight After Administration of Peplomycin

Formulation	Lymph node weight
PEP·DS[a] + mixed micelles	1.12 ± 1.51 (n = 4)
PEP + mixed micelles	3.25 ± 1.85 (n = 9)
PEP aqueous solution	3.56 ± 2.06 (n = 5)

Note: Lymph node weight in grams (Mean ± S.D.); Peplomycin (PEP) formulations administered in esophageal carcinoma rabbit model.

[a] PEP·DS is a peplomycin dextran sulfate conjugate.

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as such, can be dissociated by endogenous ionic components within the circulatory system, thereby releasing free and biologically active BLM. It is expected that the enhanced lymphatic levels of BLM provided by this delivery system should translate to an improved activity towards the treatment of lymphatic metastases. Additionally, the decreased exposure of the systemic circulation to high levels of circulating BLM would be expected to reduce the side effect profile.

An additional system we have developed and evaluated is a macromolecular complex for the lymphotropic delivery of 1-hexylcarbamoyl-5-fluorouracil (HCFU) which is a clinically useful hydrophobic prodrug of 5-fluorouracil.[53] A polymeric form of β-cyclodextrin (poly βCD) was chosen with an average molecular weight of approximately 10 kD and the hydrophobic HCFU formed an inclusion complex with the cyclodextrin (Table 2). The poly βCD was chosen as a putative lymphotropic agent, as the threshold molecular weight for the selective lymphatic delivery of dextran in the large intestine of the rat was approximately 10 kD.[40] The poly βCD-HCFU complex was water soluble and the MM system was used to promote absorption of the complex. After administration to the large intestine of the rat, the free HCFU did not exhibit any selectivity for lymphatic transport in spite of its good absorption. However, the lymphatic delivery of HCFU was increased when the combined system of the HCFU-poly βCD co-administered with MM enhancer was administered.[55]

B. TREATMENT OF LYMPHATIC METASTASIS

The chemotherapeutic treatment of esophageal cancer is very poor compared to that of other sites of cancer such as gastrointestinal cancer. In order to enhance therapy of esophageal cancer and to more safely and effectively perform esophageal cancer chemotherapy, we applied the previously described lymphotropic drug delivery system of the BLM-DS complex and the MM

TABLE 4
Histological Evaluation of Peplomycin Formulations

Dosage form	Primary tumor in esophagus		Number of metastatic lymph nodes (m = total metastasis)	
Mixed micelles + PEP·DS[a]	Moderately effective	3	Markedly effective	1
			Moderately effective	3
	Ineffective	1	Ineffective	4
	(n = 4)		(m = 8)	
Mixed micelles + PEP	Ineffective in all (n = 9)		Ineffective in all (m = 25)	
PEP aqueous solution	Ineffective in all (n = 5)		Ineffective in all (m = 12)	

Note: Evaluation of peplomycin (PEP) formulations in treatment of primary tumor and metastatic lymph node cancer in rabbit VX2 esophageal carcinoma.

[a] PEP·DS is a peplomycin dextran sulfate conjugate. Efficacy determined as markedly effective: no viable tumor cells; moderately effective: less than 1 in 3 viable tumor cells; ineffective: more than 1 in 3 viable tumor cells.

From Kuroda, D., Hamabe, Y., Ikuta, H., Katoh, M., Saitoh, Y., Yoshikawa, H., and Muranishi, S., *Drug Delivery Syst.*, 2, 107, 1989. With permission.

enhancer system to the treatment of esophageal cancer after luminal administration.[56,57] The esophageal cancer animal model possessing lymph node metastases was prepared by endoscopically implanting VX2 carcinoma into the esophageal wall of the rabbit.[57] At 16 to 18 days after implantation, rabbits were examined by esophageal contrast and those with a 1.0 to 1.5 cm tumor mass were used in the treatment experiments. In this study we chose peplomycin (PEP), a derivative of BLM, as it is more effective than BLM against squamous cell carcinoma, it is widely used in esophageal cancer chemotherapy, and it can form an ionic complex with DS. In the study, PEP was administered as either an aqueous solution with MM, or as the DS complex with the MM enhancer. Each drug formulation was intraluminally administered for 3 hr, and 10 days after treatment the primary cancer and the lymph nodes were examined. Table 3 presents the weights of the regional lymph nodes after the different treatment modalities and Table 4 describes the histological findings of the study.[57] It is well recognized in cancer therapy that lymph node weight is a good and reasonable index for the extent of lymphatic cancer metastasis. As evident in Table 3, the MM (linoleic acid-sodium taurocholate) + PEP·DS formulation was the most effective of the three formulations in depressing the normal weight increase of the lymph nodes associated with progression of the cancer. In the results of the histological study described in Table 4, although neither MM + PEP nor PEP alone demonstrated any effect against the primary tumor

or the metastatic lymph nodes, the MM + PEP·DS formulation was moderately effective in terms of treating the primary tumor, and either moderately or markedly effective in terms of treating the metastatic lymph nodes. These data suggest that absorption enhancement induced by the MM and the lymphotropic property of the DS complex[56] reinforced the antitumor activity of PEP in both the primary esophageal cancer as well as the metastatic lymph nodes. Clinical trials employing this lymphotropic delivery system are currently ongoing.

IV. SUMMARY

Although small molecular weight polar drugs are generally absorbed from the gastrointestinal tract via the portal blood, in certain instances the lymphatic transport of these drugs and macromolecules can be enhanced. This chapter has described aspects of the passage of polar drugs through the blood and lymph capillaries and has summarized the effect of molecular weight, molecular species, site of administration, and the development of lipid-surfactant mixed micelles as an enhancer system for promoting the lymphatic transport of particular macromolecules in a rat model. For small molecular weight drugs, a lymphotropic carrier system which also employed the mixed micellar enhancer system, was described as a potential means for selectively delivering these compounds to the lymphatics. These different approaches have been integrated to develop some novel lymphotropic delivery systems for anticancer agents consisting of complexes between the drug and the macromolecular lymphotropic carriers and the co-administration of mixed micellar enhancers to direct delivery towards the lymphatics after either gastrointestinal or local administration.

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Chapter 8

LYMPHATIC TRANSPORT AFTER PARENTERAL DRUG ADMINISTRATION

Yoshinobu Takakura, Mitsuru Hashida, and Hitoshi Sezaki

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I. INTRODUCTION

The lymphatic uptake of drugs after parenteral administration has largely focused on the delivery of chemotherapeutic agents for the treatment of tumor metastases, viral, and bacterial infections. Treatment for these clinical indications via the lymphatics has been investigated as the lymphatic system is an important pathway for tumor metastasis and the dissemination of viral or bacterial infection. New lesions are often formed in the lymph nodes which are not always readily accesible to chemotherapuetic agents. Therefore, to achieve effective therapy and to facilitate diagnosis, it is important to deliver chemotherapeutic agents (e.g., anticancer, antiviral, and antibacterial drugs) and radiopharmaceuticals selectively to the lymphatic system.

It is well known that macromolecules and colloidal particles such as chylomicrons, artificial oil droplets, bacteria, and red blood cells are taken up from the interstitial spaces by the lymphatic vessels but not so readily by blood capillary vessels.[1,2] This is primarily due to the anatomical and physiological differences between the blood and lymphatic capillaries. The blood capillaries have continuous basement membranes and relatively tight intercellular junctions which prevent substances from being transported into the lumen of blood capillaries from interstitial spaces. On the other hand, colloidal particles and larger molecular weight macromolecules can enter the terminal lymphatic capillaries since, generally, the basement membrane of the lymphatic capillaries is relatively poorly organized (or absent altogether) and the junctions between the endothelial cells may in places be open (see Chapter 1 for details). Therefore, one can expect that a drug could be targeted to the lymphatic system after administration to the interstitial spaces, via either intramuscular or subcutaneous injections, by employing particulates (see Chapter 9) and/or macromolecules as drug carriers.

Based on these considerations, various kinds of particulate drug carriers such as emulsions[3–10] and liposomes[11–16] have been developed as lymphatic delivery systems after topical parenteral administration. In addition to these approaches, macromolecules are of particular interest as drug carriers for lymphotropic delivery because of their diverse function and nature.[17] The purpose of this chapter is to review the different aspects of lymphatic drug delivery which have employed macromolecular carrier systems after local parenteral administration. The primary emphasis will be on the work carried out at Kyoto University although other literature examples will also be reviewed.

II. ANIMAL MODELS FOR THE ASSESSMENT OF LYMPHATIC TRANSPORT AFTER LOCAL PARENTERAL ADMINISTRATION

In order to effectively study the lymphatic transport of drugs after parenteral administration, it is necessary to choose an appropriate experimental

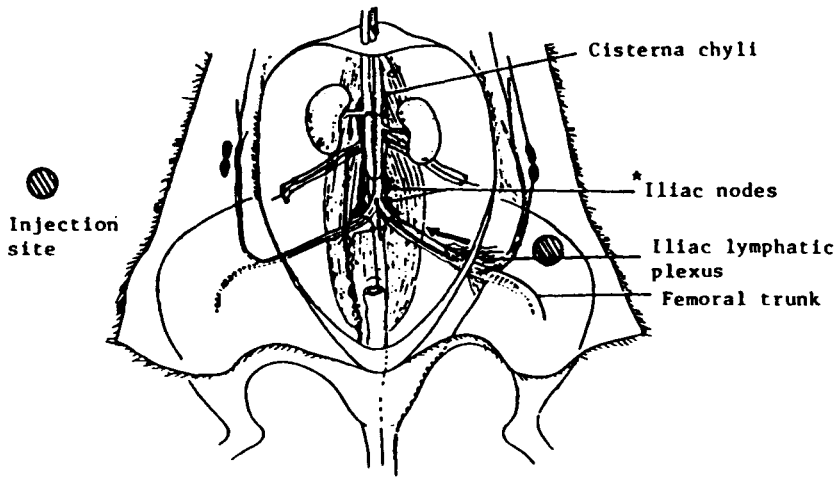

FIGURE 1. Route of lymphatic drainage from the thigh muscle in the rat. (From Tilney, N. L., *J. Anat.*, 109, 369, 1971. With permission.)

system. Generally, the lymphatic transport of a drug is evaluated by measuring the time-dependent concentration changes in lymph after administration. In rats, lymph can be collected from either the thoracic duct[18] or the intestinal lymphatics using a variety of cannulation techniques (see Chapter 3). In larger animals such as the dog[19] and sheep,[20,22] it is possible to collect peripheral lymph by cannulating the peripheral lymphatics. Drug concentration profiles in the lymph nodes can also be used for the evaluation of lymphatic transport after parenteral administration.

Figure 1 depicts a model experimental system developed for the assessment of lymphatic drug transport after intramuscular injection in rats.[1,22] Consecutive transfer of drug between the injection site (thigh muscle), regional lymph node (iliac node), thoracic lymph, and the central circulation (blood) may be readily determined in this model. The rats were given injections of drug solution into the center of the thigh muscle using a microsyringe and the levels of drug in the thigh muscle, ipsilateral iliac lymph node, and the central circulation are determined as a function of time. In this system, the thoracic duct was cannulated according to the method of Bollman and co-workers[18] under pentobarbital anesthesia. The appropriately cannulated rats were injected with a solution of the drug 30 min after surgery and were then placed in restraining cages[23] with free access to food and water. Lymph may be continuously collected for a 48-hr period.

Figure 2 shows a corresponding model for the lymphatic transport of drugs in this experimental system. The parenterally adminstered macromolecular xenobiotic could be absorbed from the injection site into either the local lymphatics or blood capillaries. After absorption directly into the peripheral lymph vessels, the drug would reach the regional lymph nodes and subse-

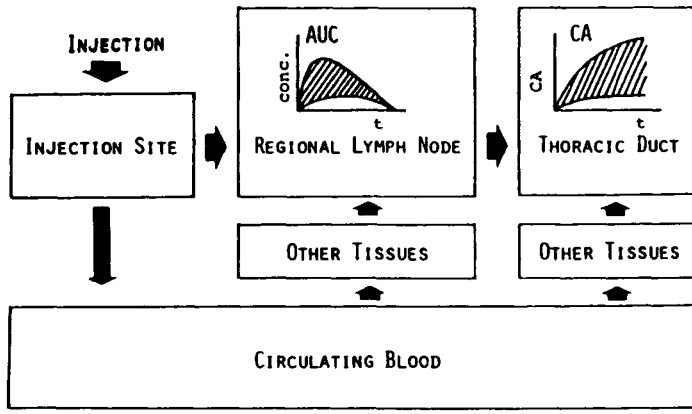

FIGURE 2. Schematic model depicting the different potential lymphatic drug transfer processes following topical parenteral administration.

quently be delivered to the thoracic duct by lymph flow. The macromolecule may also pass into the blood stream. Molecules absorbed into the general circulation from the injection site can also redistribute to the regional lymph nodes and the thoracic duct via other tissues. If necessary, the lymphatic transfer of drugs from the systemic circulation after local parenteral injection can be roughly corrected for considering lymphatic drug transfer after intravenous injection in the following manner:[5]

$$CA = CA_{i.m.} - CA_{i.v.} \tag{1}$$

$$AUC = AUC_{i.m.} - AUC_{i.v.} \tag{2}$$

$$\text{Mean Transit Time} = (AUC_{i.m.} - AUC_{i.v.})/(CA_{i.m.} - CA_{i.v.}) \times (\text{weight of lymph nodes}) \tag{3}$$

where CA is the cumulative amount of drug in thoracic lymph and AUC is an area under the drug concentration-time curve in the regional lymph node. Mean transit time is an average time necessary for transfer of drug through the regional lymph nodes and is defined by stastical moment analysis based on a unidirectional mass-transport.

Figure 3 illustrates an experimental system for conducting lymphatic absorption and transport studies after intradermal or subcutaneous administration in a conscious sheep model.[24] This system enables the collection of peripheral lymph which drains directly from the injection site. The efferent duct of the popliteal lymph node was cannulated according to the technique developed by Hall and Morris[20] for collecting the lymph from the hind limb of the sheep. A catheter in the jugular vein enabled collection of venous

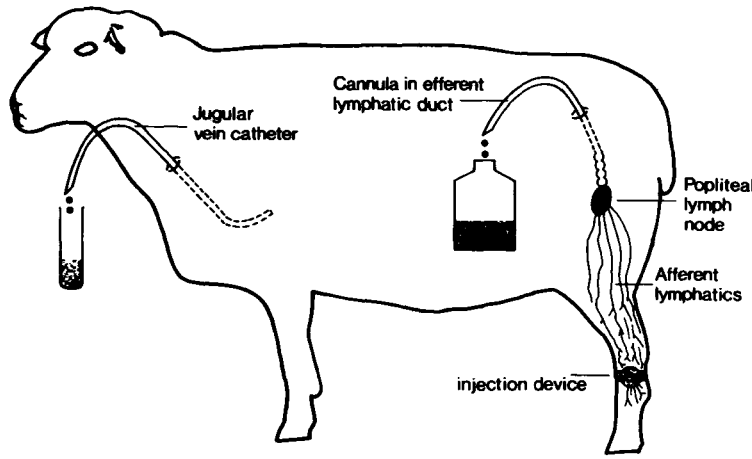

FIGURE 3. Schematic representation of the model for conducting lymphatic transport studies after parenteral administration in a conscious sheep. (From Supersaxo, A., Hein, W., Gallati, H, and Steffen, H., *Pharm. Res.*, 5, 472, 1988. With permission.)

blood samples. This model provides an excellent system by which to study the disposition of drugs in lymph and blood following topical parenteral administration.

Supersaxo and co-workers[24] reported that the cumulative recovery of human recombinant interferon alpha-2a (rIFN α-2a) in lymph following intradermal or subcutaneous administration was approximately 60% of the administered dose. These data from the sheep model indicate that it may be possible to deliver macromolecular agents which have a site of action within the local lymphatic system. In contrast, Bocci and co-workers[25] reported that the total amount of rIFN α-2a recovered in the thoracic duct lymph was less than 0.4% of the administered dose after subcutaneous injection in rabbits. The basis for these differences have not been determined although they are probably a function of the different models and the site of lymph fistulation.

III. LYMPHATIC TRANSFER OF MACROMOLECULES AFTER TOPICAL AND SYSTEMIC ADMINISTRATION

A. TOPICAL INJECTION

It is well known that the molecular size and molecular weight (MW) of water-soluble substances is a predominant factor determining the relative absorption of a compound via the blood capillary or lymphatic vessels after topical interstitial administration. It has been shown that small molecules having MW < 5 kD are absorbed primarily via the blood capillaries while compounds with MW > 20 kD appear to be cleared mainly by the lymphatics after subcutaneous or intramuscular injection.[2]

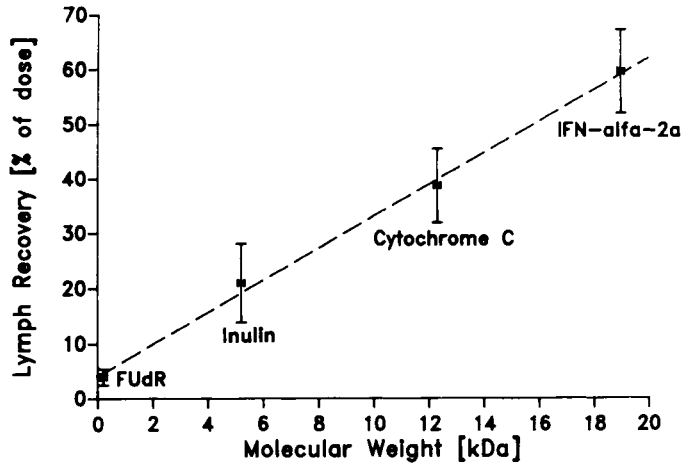

FIGURE 4. Correlation between the molecular weight and the cumulative recovery of FUdR (MW 246), inulin (MW 5,200), cytochrome c (MW 12,300) and rIFN α-2a (MW 19,000) in the efferent lymph from the right popliteal lymph node following subcutaneous administration into the lower part of the right hind leg of the sheep. Data are Mean ± S.D. (n = 3). The line was determined from linear regression and r = 0.998. (From Supersaxo, A., Hein, W. R., and Steffen, H., *Pharm. Res.*, 7, 167, 1990. With permission.)

Recently, Supersaxo and co-workers[26] studied the effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration in sheep by employing the method described in Figure 3. The cumulative recoveries in lymph draining directly from the site of subcutaneous administration were 4.0% for 5-fluoro-2′-deoxyuridine (FUdR), 21.0% for inulin, 38.6% for cytochrome c, and 59.5% for rIFN α-2a, respectively. As shown in Figure 4, there was a linear relationship between the molecular weight in the range investigated and the proportion of the dose which was lymphatically transported. The authors concluded that molecules with molecular weight larger than 16 kD are mainly transported by the lymphatics which drain the site of administration.

B. SYSTEMIC INJECTION

Lymphatic transport of drugs is often observed after intravenous administration since drugs administered directly into the systemic circulation may transfer to the lymphatic system after distribution to peripheral tissues. Figure 5 shows the tissue distribution of cationic ^{14}C-diethylaminoethyl (DEAE)-dextran and anionic ^{14}C-carboxymethyl (CM)-dextran (MW approximately 70 kD) after intravenous injection to sarcoma 180 bearing mice.[27] Significant distribution of radioactivity was observed in lymph nodes after systemic administration with the cationic compounds showing a remarkable accumulation. Similar results have also been obtained for anionic (native) bovine serum albumin (BSA) and a cationized form of BSA where an electrostatic interaction between the charged macromolecules and the lymph node tissues was sug-

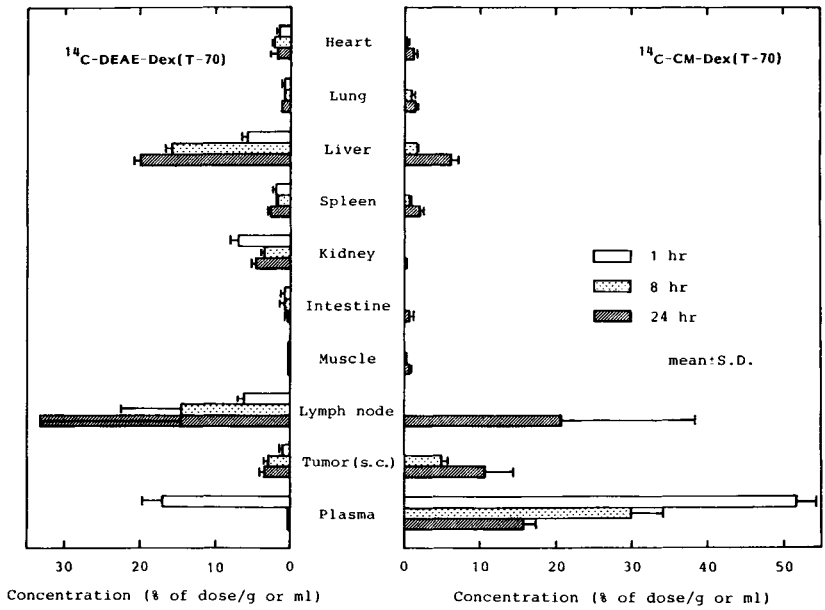

FIGURE 5. Tissue distribution of ^{14}C-DEAE-dextran(T-70) and ^{14}C-CM-dextran(T-70) after intravenous injection to sarcoma 180 bearing mice. Radioactivity in the different tissues was determined at 1, 8 and 24 hr after administration. Data are presented as Mean ± S.D. (n = 4). (From Takakura, Y., Fujita, T., Hashida, M., and Sezaki, H., *Pharm. Res.*, 7, 339, 1990. With permission.)

gested as the basis for the preferential distribution. In this study of the DEAE-dextran and CM-dextran conjugates, the molecular weight and charge characteristics of the macromolecules were demonstrated to determine their disposition profiles and were shown to strongly affect the potential targeting after systemic administration.

From these studies, it appears that lymphatic drug delivery may be achieved by utilizing macromolecules with appropriate physicochemical characteristics. However, lymphatic transfer after intravenous injection is much less efficient than direct lymphatic transport after administration as a topical injection. In addition, intravenous administration appears to result in nonspecific delivery because a molecule injected into the systemic circulation would more uniformly distribute throughout the body. Therefore, systemic administration of macromolecules has the disadvantage of not being able to direct the administered agent to the lymphatic system.

IV. LYMPHOTROPIC DELIVERY OF CHEMOTHERAPEUTIC AGENTS USING A MACROMOLECULAR PRODRUG APPROACH

In cancer chemotherapy, a sufficient supply of anticancer agents to the lymphatic system seems to offer a promising means for preventing lymph node

metastasis — a situation which leads to a poor prognosis. Advantages of the direct introduction of chemotherapeutic agents into the lymphatic vessels has been demonstrated,[28,29] but the utility of this treatment modality in surgical adjuvant chemotherapy is limited because of technical difficulties. Consequently a macromolecular carrier system may prove to be a more useful strategy for the lymphotropic delivery of anticancer agents.

Malek and co-workers first reported the use of macromolecules as a lymphotropic drug carrier system.[30] They prepared salts of antibiotic bases such as streptomycin, neomycin, viomycin, and streptothrycin with high molecular weight compounds and these macromolecular salts were termed "antibiolymphins". An increased affinity of these antibiotic salts for the lymphatics was demonstrated after intramuscular, intraperitoneal, and intrapleural adminstration. Enhanced lymphatic transfer of a bleomycin-dextran sulfate complex has also been reported from our laboratory.[31]

In addition to these noncovalent drug-macromolecule complexes, Maeda and co-workers[32,33] prepared a covalent conjugate of neocarzinostatin (NCS) with a poly(maleic acid)-styrene oligomer (SMA) for lymphotropic delivery. After subcutaneous administration of the drug-macromolecular complex (SMANCS) in rats, the drug activity was observed to accumulate primarily in the regional lymph nodes.[33] SMANCS also exhibited strong antimetastatic activity against AH109A with which the metastasis was induced.[32] The enhanced lipophilicity and increased molecular weight of the derivative appeared to be responsible for the improved activity of SMANCS relative to NCS alone.

A. DEXTRAN CONJUGATE OF MITOMYCIN C

Polymeric prodrugs of the antitumor antibiotic, mitomycin C(MMC) with dextran (Figure 6) have been developed and examined with respect to their physicochemical, pharmacodynamic, and pharmacokinetic characteristics.[34–52] The physicochemical properties of the mitomycin C-dextran conjugates (MMCD) such as molecular weight and electric charge, and the release rate of the parent compound can be controlled by manipulating the molecular size of the carrier dextran and the structure and/or the method for introducing the spacer group that couples mitomycin C to dextran. Three types of dextran having molecular weights of 10 kD (T-10), 70 kD (T-70), and 500 kD (T-500) were used as backbones and mitomycin C-dextran conjugates with cationic (MMCDcat) and anionic (MMCDan) charges were synthesized employing either ε-aminocaproic acid or 6-bromohexanoic acid as a spacer, respectively. MMCD exhibited excellent antitumor activity against not only various kinds of murine tumors,[35,36,46] but also human neoplasms.[43] Pharmacokinetic analysis revealed that MMCD acts as a macromolecular reservoir of MMC which supplies active MMC by spontaneous chemical hydrolysis in the body after systemic administration.[39] It was demonstrated that the *in vivo* disposition characteristics of MMCD could be controlled by changing the physicochemi-

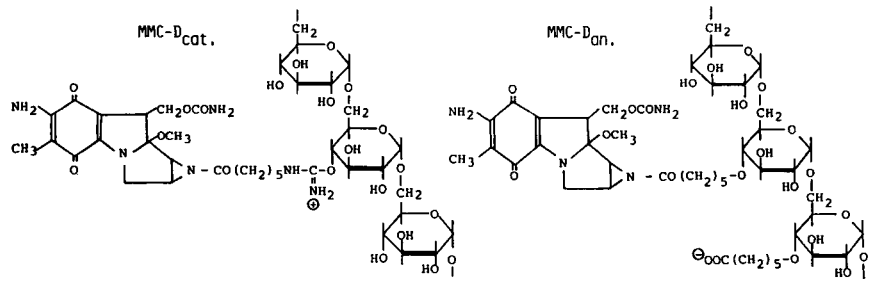

FIGURE 6. Chemical structures of mitomycin C-dextran conjugates with either cationic (MMCDcat) or anionic (MMCDan) charge characteristics.

cal properties. The usefulness of MMCDan for systemic tumor targeting has been demonstrated in mice which had been inoculated with sarcoma 180.[48]

B. DISPOSITION CHARACTERISTICS OF MITOMYCIN C-DEXTRAN CONJUGATE (MMCD) AFTER LOCAL PARENTERAL ADMINISTRATION

The absorption characteristics of MMC and MMCD following intramuscular injection were studied in rats to clarify the effect of physicochemical properties on disposition. The results were analyzed using a pharmacokinetic model and the experimental system shown in Figure 1.[40,45] Concentrations of the drug (MMC) were determined in a bioassay system (*E. coli* B was the test organism) and the carrier (^{14}C-labeled dextran) was quantified by ^{14}C analysis.

1. Disappearance from the Injection Site

The concentration profile of free MMC demonstrated a very rapid disappearance from the injection site with an apparent first order half-life of approximately 5 min (Figure 7). In contrast, MMCD exhibited slow clearance of MMC and the rate was dependent on both molecular weight and the electric charge of mitomycin C-dextran conjugate. The elimination rate of MMC administered in the form of MMCDcat was slower than observed with a MMCDan conjugate prepared from the same molecular weight dextran. The disappearance profiles of ^{14}C-dextran administered as MMCD were also determined in these studies.

Pharmacokinetic analysis was performed on these muscular absorption data using a compartmental model. The model included a Langmuir-type isotherm and is depicted in Figure 8.[45] The following assumptions were made in the development of the model: (i) MMCD is bound to the thigh muscle according to a Langmuir-type adsorption, (ii) the binding parameters do not change, (iii) only unbound MMCD is absorbed from the thigh muscle and this process follows first-order kinetics, and (iv) the conversion of MMCD to MMC proceeds at the same rate whether MMCD is bound or not, and direct inacti-

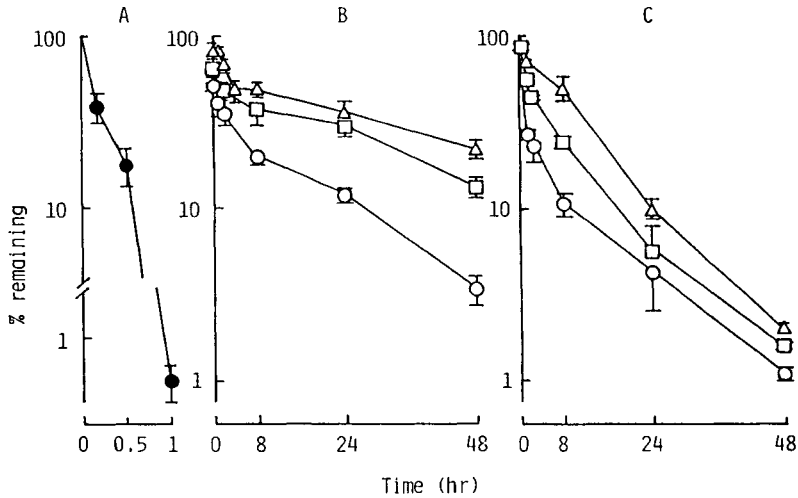

FIGURE 7. Disappearance kinetics of MMC from the thigh muscle of rats after intramuscular injection of (A) free MMC, (B) MMCDcat, or (C) MMCDan. Symbols: ●–●, MMC; ○–○, MMCDcat(T-10) or MMCDan(T-10); □–□, MMCDcat(T-70) or MMCDan(T-70); △–△, MMCDcat(T-500) or MMCDan(T-500). Data are presented as Mean ± S.D. (n = 4). (From Takakura, Y., Matsumoto, S., Hashida, M., and Sezaki, H., *Cancer Res.*, 44, 2505, 1984, and Takakura, Y., Atsumi, M., Hashida, M., and Sezaki, H., *Int. J. Pharmaceut.*, 37, 145, 1987. With permission.)

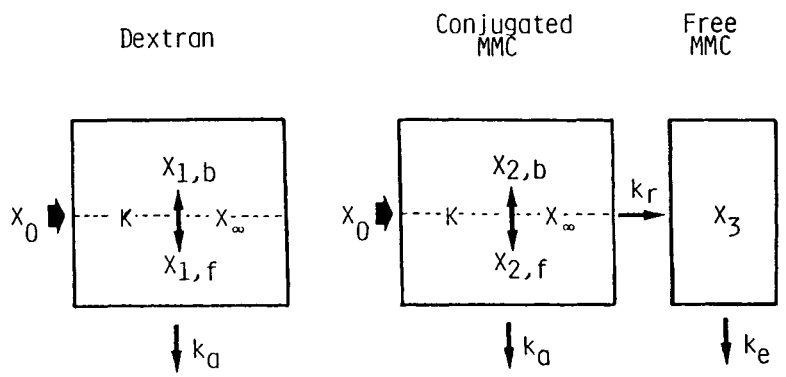

FIGURE 8. Pharmacokinetic model describing the disappearance of MMCD from the injection site after intramuscular administration in rats. (From Takakura, Y., Mori, K., Hashida, M., and Sezaki, H., *Chem. Pharm. Bull.*, 34, 1775, 1986. With permission.)

vation of MMC in the conjugated form does not occur. The following differential equations are derived from the model in Figure 8 and they predict the amount of carrier dextran in MMCD (X_1), conjugated MMC in MMCD (X_2), and regenerated free MMC (X_3) as a function of time after the intramuscular injection of MMCD.

$$dX_1/dt = - ka\ X_{1,f} \tag{4}$$

$$\begin{aligned} dX_2/dt &= - ka\ X_{2,f} - kr\ X_2 \\ &= - ka\ X_{1,f}\ X_2/X_1 - kr\ X_2 \end{aligned} \tag{5}$$

$$dX_3/dt = kr\ X_2 - ke\ X_3 \tag{6}$$

where $X_{1,f}$ and $X_{2,f}$ are the amounts of unbound carrier dextran and conjugated MMC, respectively; and ka, kr, and ke are constants describing the absorption rate of MMCD, the rate of regeneration of MMC from MMCD, and the elimination rate of free MMC, respectively. The total amount of carrier dextran (X_1) can be described by a Langmuir-type equation:

$$X_1 = X_{1,f} + X_{1,b} = X_{1,f} + X_\infty\ K\ X_{1,f}/(1 + K\ X_{1,f}) \tag{7}$$

where $X_{1,b}$, K, and X_∞ are the amount of bound carrier dextran, the binding constant, and the maximum amount of carrier dextran that can be adsorbed on the muscle, respectively. The quadratic from Eq. 7 gives:

$$X_{1,f} = 1/2\{X_1 - X_\infty - 1/K + \sqrt{(X_\infty + 1/K - X_1)^2 + 4X_1/K}\} \tag{8}$$

Substitution of Eq. 8 into Eqs. 3 and 4 yields:

$$dX_1/dt = - ka/2\{X_1 - X_\infty - 1/K + \sqrt{(X_\infty + 1/K - X_1)^2 + 4X_1/K}\} \tag{9}$$

$$\begin{aligned} dX_2/dt = &- ka/2\{X_1 - X_\infty - 1/K \\ &+ \sqrt{(X_\infty + 1/K - X_1)^2 + 4X_1/K}\}\ X_2/X_1 - kr\ X_2 \end{aligned} \tag{10}$$

The muscle clearance data of the carrier dextran and the conjugated MMC were fitted simultaneously to Eqs. 9 and 10, respectively. The computer-generated[53] parameters for MMC and MMCD are summarized in Table 1 together with those for other model macromolecules such as ^{14}C-dextran (70 kD, neutral), ^{14}C-inulin (5 kD, neutral) and ^{131}I-human serum albumin (66 kD,

TABLE 1
Disappearance Parameters of MMCDcat, MMCDan, Dextran, Inulin, and Albumin after Intramuscular Injection

	MMCDcat			MMCDan			
Parameters[a]	**T-10**	**T-70**	**T-500**	**T-70**	**Dextran**	**Inulin**	**Albumin**
ka ($hour^{-1}$)	0.878	0.702	0.395	0.741	0.776	3.68	3.66
X_∞ (% dose)	29.4	42.7	49.8	40.1	25.9	—	59.65
K ($(\%\ dose)^{-1}$)	30.4	22.0	16.1	0.763	30.7	—	1.55
kr (hr^{-1})	0.0285	0.0247	0.0198	0.0345	—	—	—
AUC (% dose · h)	2.95	4.21	5.08	2.30	—	—	—

Note: Computer-estimated parameters for disappearance from the injection site.

[a] ka: absorption rate constant; X_∞: extent of absorption; K: binding constant; kr: regeneration rate constant of MMC from MMCD; AUC: area under the curve of regenerated MMC.

From Takakura, Y., Mori, K., Hasida, M., and Sezaki, H., *Chem Pharm. Bull.*, 34, 1775, 1986; and Takakura, Y., Atsumi, M., Hasida, M., and Sezaki, H., *Int. J. Pharmaceut.*, 37, 145, 1987. With permission.

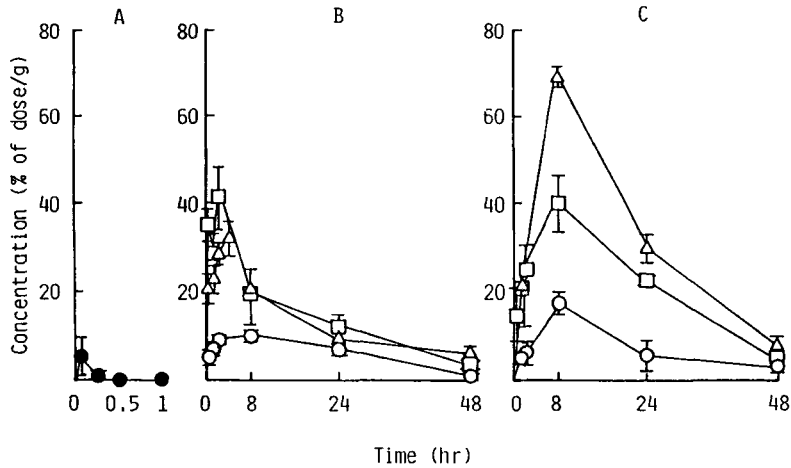

FIGURE 9. Regional iliac lymph node concentration of MMC after intramuscular injection of (A) MMC, (B) MMCDcat and (C) MMCDan in rats. Symbols: ●–●, MMC; ○–○, MMCDcat(T-10) or MMCDan(T-10); □–□, MMCDcat(T-70) or MMCDan(T-70); △–△, MMCDcat(T-500) or MMCDan(T-500). Data are presented as Mean ± S.D. (n = 4). (From Takakura, Y., Matsumoto, S., Hashida, M., and Sezaki, H., *Cancer Res.*, 44, 2505, 1984, and Takakura, Y., Atsumi, M., Hashida, M., and Sezaki, H., *Int. J. Pharmaceut.*, 37, 145, 1987. With permission.)

negative charge). The conversion rate constant of MMC (kr) was similar to the observed *in vitro* hydrolysis rate constant of MMCD at pH 7.4, thereby suggesting that hydrolysis of MMCD to MMC was the primary clearance pathway in muscle, and these data addressed the utility of MMCD as a molecular depot of MMC. The absorption rate constant was primarily dependent on the molecular weight of the administered compounds which is consistent with the data reported by Sund and Schou.[54] Furthermore, the binding constant between the conjugate and the muscle tissue was dependent upon the charge characteristics of the conjugate as MMCDcat exhibited much higher values than MMCDan(T-70). Similar drug-tissue electrostatic interactions, which can be described by a Langmuir-type adsorption isotherm, have been described for tumor cells[44] and hepatocytes[50] *in vitro*.

2. Lymphatic Transfer

After intramuscular administration, free MMC was rapidly absorbed by the blood capillaries and only trace levels of MMC were found in the lymph nodes (Figure 9) and the thoracic lymph (Figure 10).

In contrast to the free MMC data, it was demonstrated that the primary absorption route of intramuscularly injected MMCD was the lymphatic vessels. The intramuscular injection of MMCD produced significant accumulation of conjugated MMC in the regional lymph nodes (Figure 9) with subsequent transfer to the thoracic lymph (Figure 10). These results, when combined with

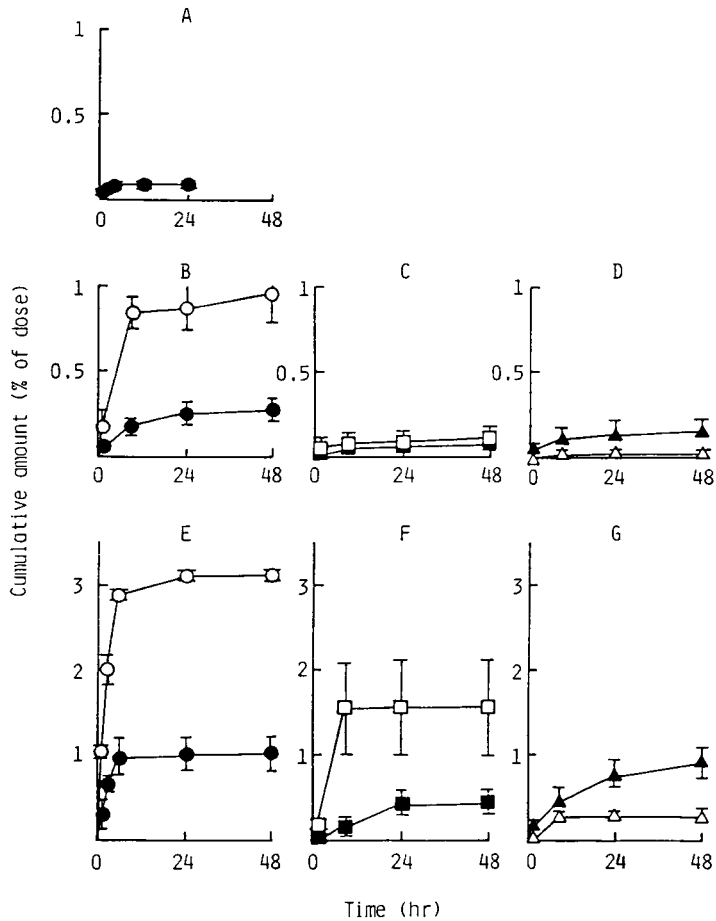

FIGURE 10. Cumulative amount of free and conjugated MMC transferred into the thoracic lymph after intramuscular injection of (A) MMC, (B) MMCDcat(T-10), (C) MMCDcat(T-70), (D) MMCDcat(T-500), (E) MMCDan(T-10), (F) MMCDan(T-70) and (G) MMCDan(T-500) in rats. Closed symbols represent free MMC concentration and open symbols represent conjugated MMC. Data are presented as Mean ± S.D. (n = 4). (From Takakura, Y., Matsumoto, S., Hashida, M., and Sezaki, H., *Cancer Res.*, 44, 2505, 1984, and Takakura, Y., Atsumi, M., Hashida, M., and Sezaki, H., *Int. J. Pharmaceut.*, 37, 145, 1987. With permission.)

the carrier ^{14}C-dextran data, indicate that the disposition characteristics in the lymphatic system after intramuscular administration depend on the physicochemical properties of MMCD. Additionally, the electric charge present on the molecule was identified as an important factor determining disposition of the macromolecular MMCD conjugates. The effect of charge on the disposition of particulates is described in Chapter 9.

MMCDcat demonstrated substantial accumulation in the regional lymph nodes although eventual transfer into the thoracic lymph was restricted. On the other hand, MMCDan passed through the regional lymph node and was readily apparent in the thoracic lymph. Furthermore, the pharmacokinetic and disposition characteristics of MMC in the lymphatic system were affected by the molecular weight of the conjugate as the smaller MMCD conjugates appeared in thoracic lymph in both the conjugated and free form, whereas the larger MMCD conjugates produced a sustained supply of free MMC to the thoracic lymph with only minimal appearance as the conjugated form (Figures 9 and 10). These results indicate that the smaller molecular weight MMCD conjugates passed through the lymph node and appeared in the thoracic lymph with the gradual liberation of MMC. The larger MMCD conjugates appeared to accumulate within the regional lymph nodes and were able to locally release the MMC.

C. LOCAL INJECTION THERAPY WITH MMCD

The local injection of mitomycin C into tissues is difficult owing to locally induced tissue damage which occurs in spite of the rapid disappearance of MMC from the injection site. These problems are of sufficient magnitude to limit the possible clinical use of mitomycin C by this route. A disposition study of MMCD after local injection revealed that derivatization of MMC into the polymeric prodrug greatly enhanced retention at the injection site and the subsequent lymphatic transport of the parent drug. Figure 11 depicts the disposition data for MMCDcat (T-500) when conjugated with 500 kD dextran. In addition to the decreased clearance of MMCD from the injection site, there was less toxicity associated with the local administration of MMCD with it being the latent form of the parent compound. These observations of decreased toxicity with MMCD have encouraged further exploration of the possible utility of MMCD for local injection therapy.

1. Intratumoral Injection Therapy

MMCDcat(T-500) was chosen for study as intratumoral injection therapy because this conjugate was found to have the longest residence period in muscle tissue (Figure 7). The retention of MMCDcat (T-500) after intratumoral injection has also been demonstrated in the tumor mass of Walker carcinosarcoma 256 implanted in the abdominal subcutaneous tissue of the rat (Figure 12) and in a VX2 carcinoma[42] subcutaneously implanted in the ear of rabbits. In normal tissue, severe necrosis was noted after the injection of free MMC whereas no side effects were observed after administration of MMCD. The intratumoral injection of MMCDcat(T-500) resulted in superior activity towards tumor growth compared to MMC in both these animal model systems. Based on these preliminary experimental results, a clinical study of MMCDcat(T-500) was initiated and the data from a study performed in more than 50 patients

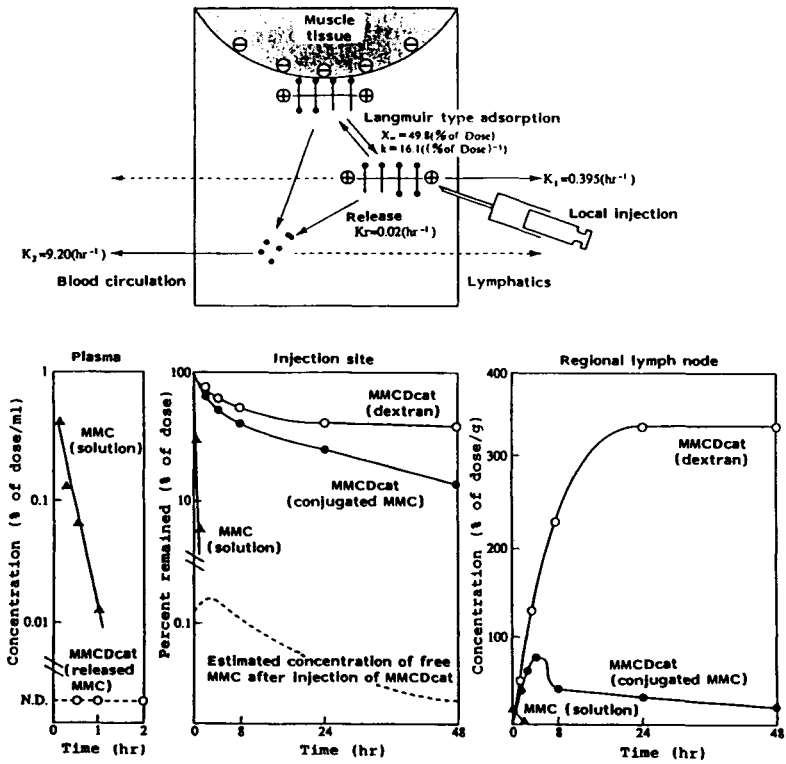

FIGURE 11. Transport profiles of MMCDcat(T-500) following topical parenteral administration.

has demonstrated the potential clinical utility of the drug conjugate without the problem of severe side effects.[43]

2. Prevention of Lymph Node Metastasis

The effect of both free MMC and MMCDcat on the progression of lymph node metastasis has been evaluated in an animal model using mouse L1210 leukemia.[55,56] In this study, L1210 leukemia cells were inoculated subcutaneously into the thigh muscle of mice and drug treatment was administered by intramuscular injection 4 days after tumor inoculation. The ipsilateral iliac lymph nodes in which the metastasized tumor cells had grown were excised and weighed to evaluate the inhibitory effect of the different drug treatments. Intramuscular injection of MMC and MMCDcat(T-10) did not show any significant effect on lymph node metastasis of the tumor which had been introduced by subcutaneous inoculation. However, treatment of the tumor-bearing mice with MMCDcat(T-70) and MMCDcat(T-500) was effective in suppressing progression of the metastasis. These observations are consistent with the lymphatic transport data in normal rats previously described in Section IV B.

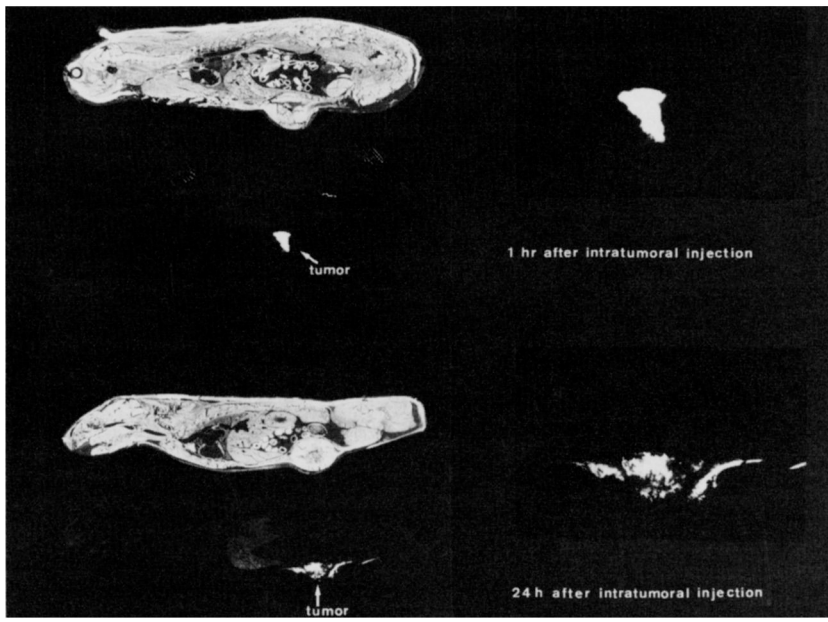

FIGURE 12. Whole body autoradiogram after intratumoral injection of ^{14}C-labeled MMCDcat(T-500) to rats bearing Walker carcinosarcoma 256. (From Honda, K., Satomura, K., Hashida, M., and Sezaki, H., *Jpn. J. Cancer Chemother.*, 12, 530, 1985. With permission.)

V. LYMPHOSCINTIGRAPHY UTILIZING MACROMOLECULAR RADIOPHARMACEUTICALS

In nuclear medicine, diagnostic lymphoscintigraphy using radiopharmaceuticals can be potentially useful in identifying regional lymph node metastasis in patients. The uptake of radiopharmaceutical imaging agents in the regional lymph nodes may indicate regional spread of metastasis and thereby improve clinical diagnosis and the subsequent treatment of the malignancy. A large number of radiopharmaceuticals have been reported for lymphoscintigraphy and these have been recently reviewed by Strand and Bergqvist.[57] Radiolabeled colloids have been used in a nonspecific manner to visualize the disruptions of lymphatic drainage from lymph nodes caused by the presence of tumor cells.[57] Nonspecific macromolecules such as albumin[58] and dextran[59] have also been employed for the same purpose.

Among the many different radiopharmaceuticals which have been employed for lymphoscintigraphy, radiolabeled antibodies which can specifically bind to antigens on the surface of target cells appear to be a promising approach to therapy, and this approach is generally termed "immunolymphoscintigraphy".[57] The radiolabeled antibodies are most often administered intravenously for the diagnosis and therapy of tumors. However, if the location of the target tumor is known, then local injection would potentially offer many

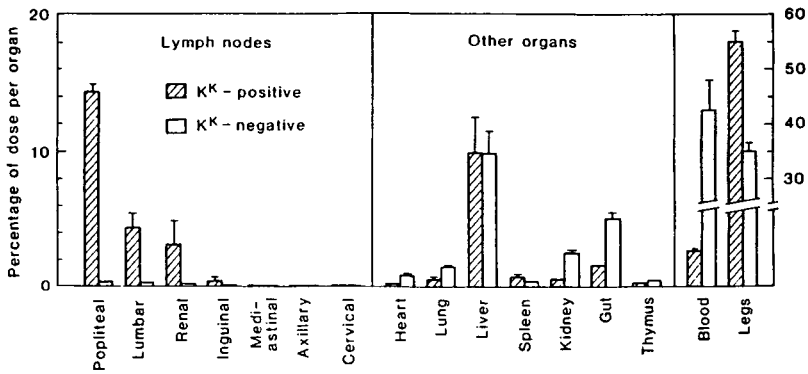

FIGURE 13. Distribution of ^{125}I-labeled antibody in lymph nodes and different organs and tissues 2 hr after administration to H-2K^K positive or H-2K^K negative mice. Administration of the radiolabeled antibody was via subcutaneous injection into the hind leg food pad. Data are presented as Mean ± S.E. (n = 3). (From Weintein, J. N., Parker, R. J., Keenan, A. M., Dower, S. K., Morse, H. C., and Sieber, S. M., *Science*, 218, 1334, 1982. With permission.)

therapeutic advantages over the less specific intravenous therapy. The relative permeability characteristics of the blood and lymphatic capillaries play a role in the localization of subcutaneously administered colloidal systems. For example, immunoglobulins such as IgG (150 kD) and IgM (900 kD) are preferentially transported by the lymphatics when injected into interstitial space, due to the anatomical characteristics of the lymphatic capillaries.[1] Then they traverse the regional lymph nodes before entering the blood stream via the thoracic lymph duct (Figure 2). It is during the transit through the regional lymph nodes that they have the possibility to specifically associate with the target cells. Polyclonal antibodies directed against tumor-associated antigens, such as ferritin[60] and carcinoembryoric antigen (CEA),[61] have been injected locally into patients for imaging of tumors present within lymph nodes.

Weinstein and co-workers[62–64] demonstrated that subcutaneously administered monoclonal antibodies could localize within regional lymph nodes and specifically label target cells using a monoclonal antibody directed against the normal cells. These workers studied the disposition of a murine monoclonal IgG2a antibody which is specific for H-2K^K which is the mouse class I major histocompatability antigen and the pertinent data are presented in Figure 13. The monoclonal antibody was injected into the hind footpads of mice which were expressing the antigen and 10 to 20% of the injected dose could be found in the ipsilateral popliteal node 2 hr after administration. Importantly, the antibody uptake in the popliteal nodes of H-2K^K-negative mice was 50-fold less than in the H-2K^K-positive mice. When the antibodies were administered intravenously, only trace amounts of the injected radioactivity were present in the popliteal nodes of either the antigen-positive or -negative mice. This same

group has evaluated the selective lymphatic delivery of monoclonal antibodies where the disposition of a monoclonal IgG1 antibody directed against a specific antigen on line 10 hepatocarcinoma of guinea pigs was studied.[63,65] The results from these studies indicated a specific distribution of the monoclonal antibody to the metastasized tumor cells present in the regional lymph node of the animals following subcutaneous injection.

In addition to these animal studies, the utility of monoclonal antibodies for immunoscintigraphy has been evaluated in a number of different clinical situations. For example, Thompson and co-workers[66] demonstrated that lymph node metastasis in breast cancer patients could be detected after the subcutaneous injection of a ^{131}I-labeled monoclonal antibody. Furthermore, a phase 1 clinical study conducted using a monoclonal antimelanoma antibody (a DTPA conjugate labeled with ^{111}In) for immunolymphoscintigraphy in patients with metastatic melanoma revealed preferential localization of the ^{111}In activity in the metastasized lymph nodes of a patient with both tumor-bearing and tumor-free lymph nodes.[67]

VI. SUMMARY

Various aspects of the lymphatic delivery of chemotherapeutic agents using macromolecular carrier systems after parenteral administration have been reviewed. This approach has been shown to be a useful strategy for both the treatment and diagnosis of diseases present in the lymphatic system, and the results of animal and clinical studies appear to indicate a particular utility for the treatment of lymph node metastasis. Therefore, the parenteral administration of macromolecular carrier systems appears to be a promising tool for lymphatic drug delivery to metastatic tumors. This concept also has application to the treatment of other disease states (e.g., viral, bacterial, and fungal infections) and pathological conditions.

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Chapter 9

PARTICULATES AND LYMPHATIC DRUG DELIVERY

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I. INTRODUCTION

It has long been known that macromolecules absorbed intact across the gastrointestinal tract (GIT) mainly reach the systemic circulation through the lymphatics. For example, May[1] showed that *Clostridium botulinum* type A toxin (900 kD) was absorbed mostly through the lymph after oral administration in rats and rabbits. However, intestinally absorbed macromolecules do not pass exclusively into the lymphatics. Warshaw et al.[2] studied the intestinal absorption of horse radish peroxidase (HRP, 40 kD) and concluded that small but significant amounts of the tracer were consistently transmitted across the gut into the intestinal lymph and the portal blood. The proportion of an absorbed macromolecule that enters the lymphatics can vary considerably and is dependent upon the physicochemical properties of the macromolecule.[3,4]

Since the rate of lymph flow in the intestine is approximately 500 times less than that of blood flow in the portal vein, unless selective uptake into the lymphatics occurs, most drugs will be transported almost exclusively by the portal circulation. One of the factors favoring lymphatic transport is increasing lipophilicity, another is increasing molecular size. The lymphatic vessels are adapted for the absorption of large macromolecules and particulates, so it is highly likely that particulates taken up across the GIT will gain access to the local lymphatics. In recent years, experimental evidence has accumulated to indicate that particulates can be taken up across an intact GIT.[5] However, despite the appearance of convincing evidence, the intestinal uptake of particulates remains a controversial subject which is still viewed with scepticism by many. Perhaps doubts and uncertainty are encouraged by some poorly designed studies which have claimed remarkably high levels of uptake and/or extensive penetration of particulates into deep body sites. In addition, the scepticism surrounding this subject is probably enhanced by the descriptions of

TABLE 1
Proposed Mechanisms of Uptake of Particulates Across the Gastrointestinal Tract

Site/mechanism	Size range	Fate after uptake	Ref.
Enterocyte/endocytosis	< 220 nm	Reticuloendothelial system (RES)	9 – 13
Paracellular transport	100 – 200 nm	Not studied	19
Intestinal macrophages	~ 1 mm	Mesenteric lymph nodes	20, 21
'Persorption'	5–150 mm	Blood and excretory fluids	23, 24
Peyer's patches	20 nm – 10 mm	Mesenteric lymph nodes and RES	26 – 36, 39 41 – 44

several alternative methods of uptake proposed by different authors[5] (Table 1). Some of the methods described appear physiologically unsound and quite unconvincing. Nevertheless, poorly accepted and unsubstantiated claims should not be allowed to detract from reports which have elegantly and unequivocally demonstrated the uptake of particulates across the GIT.

The uptake of particulates across the GIT is an important phenomena which may have toxicological, immunological, pathological, and drug delivery implications for humans and other species, especially in view of the extensive particulate contamination of the environment and the abundance of various particulates in many diets. Important considerations include the nature, size, and shape of particulates that can penetrate the GIT, the frequency of uptake, and their eventual fate. However, such considerations are beyond the scope of this chapter. Here we are concerned with the potential use of particulates as carrier systems for lymphatic delivery following oral administration. The various proposed mechanisms of uptake of particulates across the GIT will be discussed and their eventual fate will be considered.

Although some small molecular weight lipoidal molecules such as DDT[6] have been shown to be selectively transported into the lymph and the extent of absorption can be influenced by the vehicle in which the drug is administered, most drugs are not sufficiently lipophilic to be lymphatically absorbed. Consequently, various lipid-based delivery systems such as emulsions, liposomes, and micelles have been employed in attempts to promote lymphatic absorption following oral administration. The data on the use of such colloidal delivery systems to promote lymphatic absorption of drugs and macromolecules will be reviewed.

One of the principal functions of the lymphatic system is to recirculate the interstitial fluid to the bloodstream, clearing foreign matter, particulates, and dead cells, and thus maintaining an optimum cellular micro-environment.[7] Particulate drug delivery systems administered parenterally at peripheral sites therefore present an opportunity for selective lymphatic delivery. Although such delivery systems would be of interest in the treatment of all lymphatic diseases, most work in this area has concentrated on the detection and eradication of lymph node metastases. At present, surgical removal of a primary

tumor is usually followed by the intravenous (IV) administration of highly cytotoxic anticancer agents. IV administration results in only a tiny percentage of the injected drug reaching the target site, while the majority of the dose causes classical chemotherapeutic side effects in nontarget tissues. Selective lymphatic delivery of drugs in suitable particulate systems could eliminate most of these side effects and increase drug delivery to the target site.

Therefore, as well as oral uptake pathways, this chapter will also discuss the mechanisms by which particulates are taken up into, and distributed through, the lymphatic system after local injection. The effects of the physicochemical properties of the particulate on these processes will also be considered. A critical review of the particulate delivery systems which have so far been used for parenteral lymphatic targeting will also be presented.

II. PROPOSED MECHANISMS OF UPTAKE OF PARTICULATES ACROSS THE GASTROINTESTINAL TRACT

A. ENDOCYTOSIS BY ENTEROCYTES

It is now firmly established that intestinal uptake of intact macromolecules by enterocytes occurs to a limited extent in adults of most species studied.[8] Since the molecular size of large macromolecules overlaps the molecular size of small particulates, it is perhaps not surprising that the uptake of particulates by enterocytes has also been reported on a number of occasions. For example, the pinocytotic uptake of azo dye particles (20 to 40 nm) by rat duodenal cells was reported by Barrnet in 1959.[9] The particles were identified in membranous vesicles which laterally migrated from a supranuclear position and released the particles into the intercellular space. In a later study involving oral feeding of particulates to rats, Sanders and Ashworth[10] demonstrated the uptake of latex particles (220 nm) into jejunal enterocytes. The particles were carried through the cytoplasm in intact vesicles, discharged into the lamina propria, and gained access to the lymphatics.

Worthington and Syrotuck[11] investigated the intestinal permeability to particulates in both normal and protein deficient rats. Ferritin (12 nm) and adenovirus type 5 were pinocytosed into jejunal enterocytes and were believed to be present in lysosomal bodies of typical absorptive cells. Furthermore, the uptake of cationized ferritin by the colonic epithelium of guinea pigs, rats, and mice was reported by Barbour and Hopwood;[12] the particles were identified in membranous vesicles and subsequently in the secondary lysosomes.

More recently, the uptake of Percoll microspheres (28 to 30 nm) by enterocytes of suckling mice following repeated oral gavage was demonstrated by Matsuno et al.[13] Large quantities of particles were identified in ileal enterocytes, mainly in large apical vacoules, but were also evident in membrane-bound small vesicles and in secondary lysosomes. It has been well established that the intestinal epithelium of newborn mammals is a less effective barrier to macromolecules and particulates than the epithelium of adult animals.[14] A recent study compared the uptake of particulate manganese oxide

(1 μm) in pre-weanling and weaned rats and it was demonstrated that particulate uptake was dose and age dependent, with greater uptake occurring in the pre-weanling animals.[15]

Hence, the available evidence indicates that small particulates can be taken up across both mature and immature intestinal epithelium by a pinocytotic mechanism involving ordinary enterocytes. The upper size limit for uptake by this mechanism is unknown, but the reported uptake of particulates as large as 220 nm is perhaps surprising.[10] The extent of uptake by this route is also unknown as none of the described studies were designed to be quantitative. Nevertheless, it seems likely that the uptake of particles by enterocytes is relatively infrequent and does not prevent the intestinal epithelium from acting as an effective barrier to limit the absorption of macromolecules. Therefore, it appears unlikely that this uptake mechanism could be exploited for drug delivery purposes.

B. PARACELLULAR TRANSPORT

In normal circumstances, the zona occludens (or tight junctions) of the epithelial cell membrane is thought to act as an effective barrier to all but the smallest of molecules. This barrier function has been extensively investigated in a number of studies involving several macromolecular tracers. For example, Madara and Trier[16] used three tracers (1.9, 12.3, and 40 kD) to investigate the permeability of the tight junctions between adjacent epithelial cells in the rat small intestine. The tracers were not observed to penetrate the tight junctions between undamaged absorptive cells or goblet cells. Similiarly, Yamamoto[17] found that HRP and ferritin were excluded from the tight junctions of rat jejunum.

In view of these reports and others, the passage of particulates between absorptive cells (paracellular transport) seems less likely than transport through cells. However, the paracellular transport of polyalkylcyanoacrylate nanocapsules (100 to 200 nm) has been reported in beagle dogs by Aprahamian et al.[18] The nanocapsules contained an iodinated oil (lipiodol) and uptake of lipiodol emulsion alone was also investigated. Cryofixation and freeze fracture methods coupled with X-ray microprofile analysis in a scanning electron microscope suggested that the nanoparticles were taken up intact and reached the vascular compartment. This is surprising, since one would imagine that the lymphatics would be more likely to be involved in the transport of such particulates. A few small emulsion droplets were found in the lymphatics, but it was questionable whether these had been transported transmucosally (Section IV B.2). It was reported that nanocapsules were never detected in the cytoplasm of enterocytes, but were found in larger defects of the mucosa. Passage through defects may have been an important route, since damage to the mucosa may have been induced by the presence of a surfactant (Pluronic F68). Nevertheless, the paracellular route of transport was emphasized by Aprahamian et al.[18] The nanocapsules were prepared in the presence of 0.5% Pluronic F68 and the colloidal preparation was concentrated before administration. The possibility of damage to the mucosa caused by the surfactant should not be

ignored and may influence the mechanism of particle uptake. No quantitative determination of the extent of uptake of the nanocapsules was possible in the study by Aprahamian et al.[18]

Only one other report describing the paracellular passage of particulates in the intestine appears to be available. Worthington and Syrotuck[11] reported the presence of intracellular ferritin in the intestinal epithelium of rats subjected to a protein deficient diet. However, these animals displayed deterioration of junctional complexes as a result of their diet and particles were not observed in the intercellular spaces of animals fed a normal diet.

Work from our laboratory with poly (butyl-2-cyanoacrylate) nanoparticles (600 nm) has indicated that these particles are taken up by the M cells of rat Peyer's patches (PP), but not by other cells (Figure 1). Aprahamian et al.[18] did not study uptake into the PP. Neverthless, despite doubts about the mechanism of uptake of these nanocapsules, uptake must have occurred as evidenced by a biological response to an entrapped peptide following oral administration.[19] These findings have recently been confirmed in a subsequent study by the same group, in which nanocapsules were administered at different sites of the intestine of rats and the extent of induced hypoglycemia was determined.[19a] The ileum was reported to be the best site for absorption of nanocapsules.[19a]

C. INTESTINAL MACROPHAGES

It has been proposed by Wells et al.[20] that macrophages may play a role in the intestinal uptake of particulates. They hypothesized that particles could be phagocytosed by gut macrophages within the intestinal wall and pass intracellularly to the mesenteric lymph nodes. In a preliminary study to test this hypothesis in mice, they demonstrated uptake and transport to the mesenteric lymph nodes (MLN) of fluorescent particles (1 μm) after oral administration.[20] The particles were detected exclusively in the macrophage population of the MLN and were not found in the lymphocyte population.

A subsequent study documented the uptake of fluorescent particles of two different colors and sizes (0.85 and 1.09 μm) across independent jejunal loops in mongrel dogs and ileal loops in rats.[21] MLN macrophages contained multiple particles of one or the other color, indicating that intestinal phagocytes transported the particles to the draining lymph nodes after phagocytosis in the intestine. However, it was not clear which cells were responsible for the initial phagocytosis and at which site, within or above the intestinal epithelium.

The phagocytic uptake of the protozoa *Giardia muris* by macrophages in the intestine of mice has been demonstrated by Owen et al.[22] Macrophages beneath the basal lamina propria in PP extended pseudopodia into the epithelium to trap invading organisms and enclosed them in phagolysosomes. This mechanism of uptake may be similiar to that described for particulates by Wells et al.[21] Although the intestinal segments used by Wells et al.[21] were free of grossly visible PP, the presence of isolated lymphoid follicles could not be excluded.

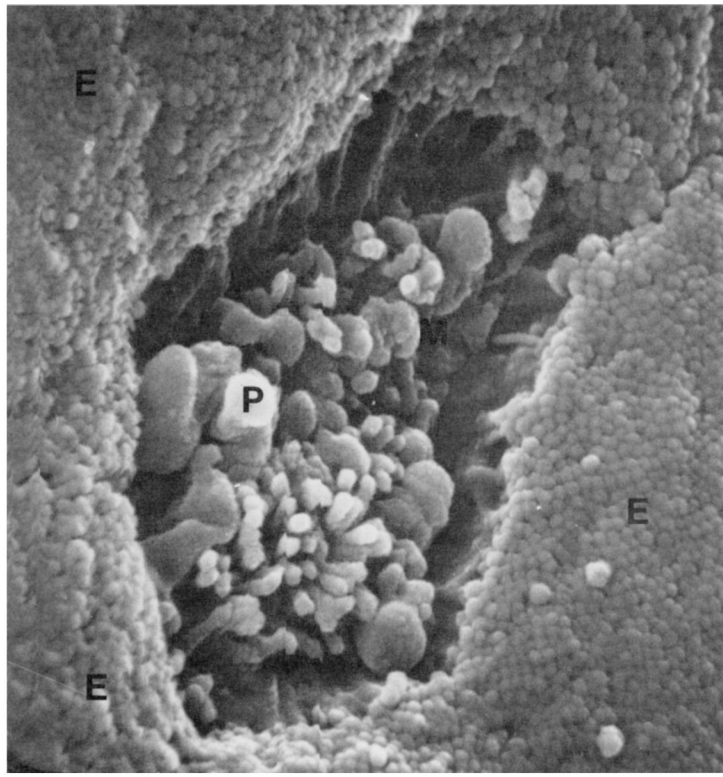

FIGURE 1. Scanning electron micrograph of a single poly (butyl-2-cyanoacrylate) particle of 600 nm diameter (P) on the surface of an M cell (M) displaying typical microfolds. The M cell is surrounded by ordinary enterocytes (E) showing normal "finger-like" microvilli. Rat Peyer's patch × 14,500. (Micrograph kindly produced by Dr W. Sass, Department of Experimental Surgery, Christian-Albrechts-University Kiel, Germany.)

D. PERSORPTION

It was first reported in the last century that large, orally ingested, solid, nondeformable particles were somehow able to penetrate the GIT and appear in the bloodstream. This phenomenon was termed "persorption" and it has been extensively studied more recently by Volkheimer and co-workers. In the studies that Volkheimer performed on himself,[23] a wide range of particulates such as diatoms, pollen, spores, and starch granules were reported to be regularly demonstrable in blood and urine after oral administration. Furthermore, in experimental studies in animals, a wide variety of particulates was reportedly found in various body fluids after oral administration, including urine and cerebrospinal fluid.[24] The persorbability of particulates was reportedly limited by their size and hardness, with the best results being obtained for particulates of 7 to 70 μm. For polyvinylchloride particles, the upper limit for

persorbability was 150 μm. Persorbed particles were transported both in the chyle and the portal circulation and large particles were found in the circulation several hours after administration.[23,24] Such extraordinary findings appear to be seriously at odds with established physiological and anatomical principles. The physiological fate of particulate carriers of a variety of compositions and size ranges following parenteral administration has been well documented.[25] It seems inconceivable that even if such large particulates were able to penetrate the GIT, perhaps through breaks in the epithelium, that they would be capable of distributing as widely as Volkheimer suggested.

Since Volkheimers' work, many additional research groups have extensively investigated the intestinal uptake of particulates, using more sophisticated analytical procedures and improved experimental methodologies. For example, Le Fevre et al.[26] assessed the uptake of 15.8 and 5.7 μm particles after acute and chronic oral administration to mice. There was no evidence for the uptake of the 15.8 μm particles, while the 5.7 μm particles accumulated in the PP during chronic feeding. However, none were found in the blood and few were found in the liver. Hence, the results of Volkheimer must be viewed with considerable scepticism, since the vast majority of evidence indicates that the uptake of particles of greater than 10 μm across a normal intestinal epithelium is unlikely.

E. PEYER'S PATCHES

1. Chronic Uptake Studies

In a series of chronic feeding studies, Joel and Le Fevre[27] repeatedly demonstrated the uptake and accumulation of colloidal carbon and polymeric particles (latex) in the PP of experimental mice. The uptake was shown to be cumulative, since particles could be identified with difficulty after only 2 days, but with ease after 2 months. Although the PP comprised only a small fraction of the total weight of the intestine, they contained the majority of the particles that were taken up. Particle uptake showed a nonlinear dependence on the concentrations ingested and it was demonstrated that isolated lymphoid follicles were also capable of uptake.[28] Several attempts were made to quantify the extent of particle uptake, and although this proved experimentally difficult the estimates of the extent of uptake were generally low.[29] For example, it was estimated that less than 0.01% and 0.0055% of single administered doses of latex (170 to 250 nm)[30] and carbon (27 nm)[31] were taken up across the intestine, respectively. There appeared to be a marked difference in the ability of particulates of similiar sizes to accumulate in PP and the evidence suggested that hydrophobic particles were taken up more readily than hydrophilic particles.[32,33]

Recently, Le Fevre et al.[34] used fluorescent particles (1.8 μm) to compare the extent of uptake in young and aged mice. Surprisingly, greater uptake was observed in aged mice, though this may have been as a result of the more rapid clearance of particles from the PP of young mice. Uptake and accumulation of

fluorescent particles (100, 500 nm, 1 and 3 μm) in the PP of rats has also been demonstrated by Jani et al.[35] The smaller particles were taken up more efficiently than the larger ones and nonionic particles were taken up to a greater extent than carboxylated particles.

Eldridge et al.[33] used a range of different particles with an entrapped fluorescent dye to investigate particle uptake in mice. Only particles of 1 to 10 μm achieved uptake and a rank order of uptake was established dependent on increasing hydrophobicity. Ebel[36] described a method for quantifying the extent of absorption of model fluorescent particles from the mouse small intestine. Although this technique counted 94 ± 8% of particles added to tissue specimens, a complete determination of particle uptake was not possible because the full length of the intestine was not included in the counting procedure. Nevertheless, the technique did provide accurate data on uptake and demonstrated that it was dependent on particle size, dose level and the fed state of the animal. The results of Ebel[36] indicated that less than 0.01% of the 10^6 to 10^8 administered particles were taken up.

A recent report by Alpar et al.[37] claimed that the extent of particle uptake in rats was several orders of magnitude greater than had been previously reported. It was calculated that about 39% of an orally administered dose of microparticles (1.1 μm) was circulating in the blood 45 min after administration. The mechanism responsible for this phenomenally rapid and extensive uptake of particles was not described, nor was the mechanism of evasion of the RES, which would normally be expected to rapidly phagocytose circulating particles. Similiar startling results were reported in a subsequent study involving oral administration of albumin microparticles (1 to 4 μm) where 35 to 37% of the administered dose was calculated to be circulating in peripheral blood 60 min after administration.[38] In addition, Florence et al.[39] have also recently reported a very high extent of uptake of particulates across the gastrointestinal tract in rats. Following oral intubation of polystyrene microparticles for 10 days, tissue analysis for the presence of polystyrene by gel permeation chromatography indicated that the extent of uptake of particles was about 34% for 50 nm particles and 26% for 100 nm particles. Particles were recovered from the liver, spleen, blood, kidneys, and bone marrow in varying quantities, but particles greater than 100 nm did not reach the bone marrow and those larger than 300 nm were absent from blood.[39] Although the extent of uptake reported by Florence et al.[39] is less than that reported by Alpar et al.,[37,38] it is still extraordinarily high in comparison to alternative studies. Studies in our laboratories on the uptake of particles of similiar size across the intestine of rats suggest a very low level of uptake (unpublished observations).

In summary, although many studies have independently demonstrated the uptake of particles following oral administration, the extent of uptake remains poorly defined and controversial. While Alpar et al.[37,38] and Florence et al.,[39] have recently reported extensive uptake, alternative groups, particularly Le Fevre and Joel,[27] have consistently reported much lower levels of uptake.

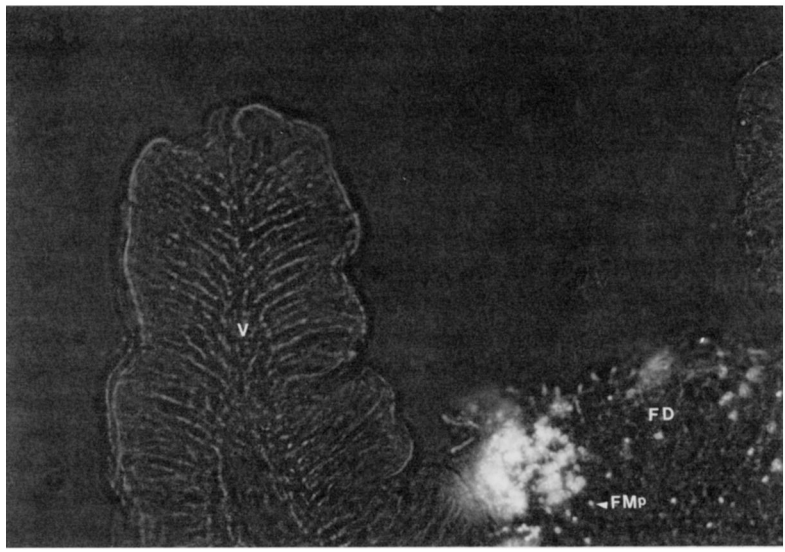

FIGURE 2. Photomicrograph of a lymphoid follicle dome (FD) showing extensive uptake of fluorescent latex microparticles (FMp) (0.94 μm). No particle uptake was observed into the adjacent villi (V). Rabbit Peyer's patch ×100. (Micrograph kindly produced by K. Howard and Dr N. Thomas, Department of Human Morphology, Queen's Medical Centre, Nottingham.)

2. Direct Demonstration of Endocytosis by the Follicle Associated Epithelium

Although not directly demonstrated, Le Fevre and Joel suggested that M cells in the follicle associated epithelium (FAE) of the PP were responsible for the uptake of particles.[27] The uptake of fluorescent microparticles by Peyer's patch epithelial cells has been demonstrated in studies performed at Nottingham and are depicted in Figure 2. M cells are specialized cells of the FAE (Figure 3) which have been ascribed an antigen sampling function and have been shown to be responsible for the uptake of macromolecules, bacteria, viruses, and other pathogens across the intestine (Table 2).[40] Two important implications of M cell transport are (i) material endocytosed will probably escape degradation and, (ii) material will be released into an environment rich in immunocompetent cells.

Seifert et al.[41,41a] demonstrated the uptake of latex particles (0.5 μm) by M cells in Wistar rats. Under scanning electron microscopy, particles were readily identified on M cells, usually surrounded by microfolds, from where they were rapidly taken up and found within macrophages in the PP.[41a] Jeurissen et al.[42] also reported the uptake of native and HRP-coated latex particles (0.48 μm) by M cells in rats and mice. In addition, Pappo and Ermak[43] demonstrated the uptake of fluorescent latex particles (600 to 700 nm) by M cells in rabbits. It

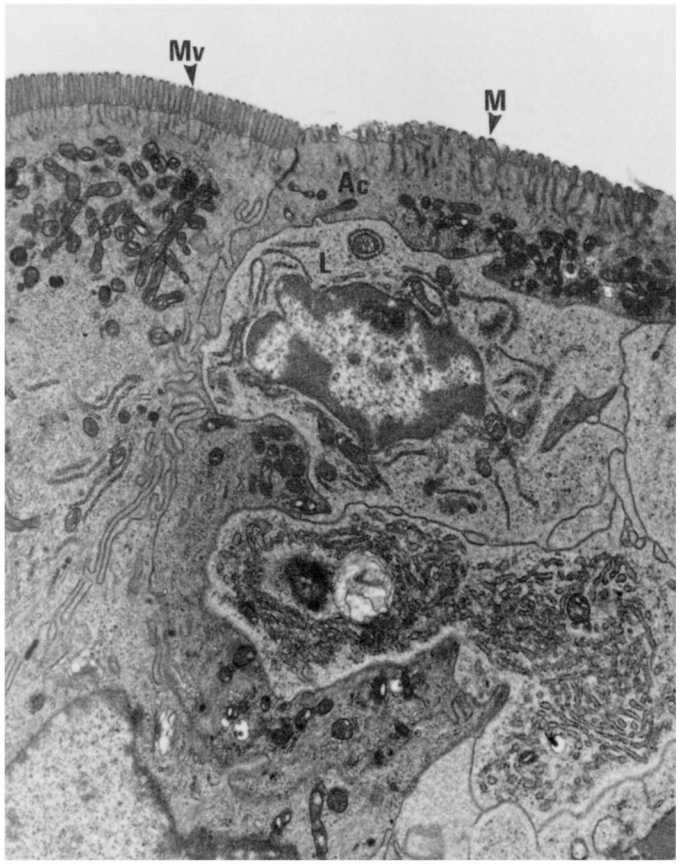

FIGURE 3. Transmission electron micrograph of an M cell (M) in the follicle associated epithelium of a Peyer's patch. Characteristic features include; stunted microvilli compared to the adjacent enterocyte (Mv), short apical cytoplasm (Ac) and the presence of an enclosed lymphocyte in the intercellular pocket of the M cell (L). Rat Peyer's patch × 7,690. (Micrograph kindly produced by K. Howard and Dr N. Thomas, Department of Human Morphology, Queen's Medical Centre, Nottingham.)

was reported that the extent of uptake into rabbit PP was at least one order of magnitude greater than uptake into rats.[43] Furthermore, it was calculated that approximately 5% of the intraluminal dose of particles was taken up into the rabbit PP in this study.[43]

Landsverk[44] reported the uptake of latex particles (250 and 610 nm) by the FAE of ileal PP in 3- to 4-week old calves, but M cells were not thought to be responsible. The particles were embraced by surface protrusions which extended from the concentric folds of the FAE and were internalized into cytoplasmic vacuoles.

TABLE 2
Materials and Micro-organisms Known to be Transported Across the Intestine Through M Cells or Follicle-Associated Epithelial Cells

Macromolecules	Bacteria	Viruses	Protozoa	Particulates
Native ferritin	Mycobacteria	Reovirus	Cryptosporidia	Latex
Cationized ferritin	Vibrio cholerae	Rotavirus		Carbon
Horseradish peroxidase	Campylobacter jejuni	Poliovirus		Poly (lactide-co-glycolide)
Ricinus communis agglutinin	Chlamydia	Parapox virus		Poly (butyl-2-cyanoacrylate)
Wheat germ agglutinin	Shigella flexneri			Horseradish peroxidase on latex
Cholera toxin				
Immunoglobulins				

III. FATE OF PARTICULATES AFTER INTESTINAL UPTAKE

With the notable exception of one or two rather inexplicable claims, such as the uptake of very large particulates[23] or the rapid appearance of many smaller particles in peripheral blood,[37] the reports of uptake of particulates across the GIT seem to agree on the subsequent fate of the particles. Initially, particles appear almost exclusively in the lymph, probably intracellularly, and only limited penetration to alternative body sites occurs. The transport of particles from the PP to the mesenteric lymph is delayed, a finding which has been recently confirmed in studies[45] on the intestinal absorption of a 55 kD macromolecule, poly (d-lysine), (PDL). Ho et al.[45] demonstrated that PDL was extensively endocytosed by PP epithelial cells in rabbits and subsequently released into the submucosa. The PDL was then trapped in lymphoid follicles and the rate-determining step for the appearance of PDL in the lymph was migration of the lymphocytes.[45]

Sanders and Ashworth[10] observed particles (220 nm) in the interstices of the lamina propria and in the lymphatics of the mucosa 2 to 4 hr after administration. Subsequently, they were observed in the hepatic sinusoids and were especially numerous in the spaces between the endothelial lining and the hepatic cells (space of Disse). In animals that had ingested the particles for 2 and 4 days, the particles were found widely distributed in the liver cell cytoplasm. These findings were in general agreement with those of Matsuno et al.[13] who suggested that orally ingested Percoll microspheres (28 to 30 nm) entered the general circulation through lymphatic drainage of the intestine. Small numbers of particles were found in the Kupffer cells of the liver. Lymphatic transport was demonstrated by the appearance of microspheres in macrophages

located in the MLN and the possibility of further transport by circulating macrophages was raised.

The transport of intestinally derived fluorescent (1 μm) microparticles in the mesenteric lymph has been well documented by Wells et al.[20,21] Intestinal macrophages were shown to be responsible for the transport of particles to the MLN, but further penetration by the particles was not studied. The fate of particles was studied in the chronic feeding studies of Le Fevre and Joel,[27] but they found little evidence for significant transport of particles from the PP to the bloodstream. After uptake, particles were phagocytosed by macrophages, which were thought to be responsible for particle transport from the dome to distant areas of the patch. Particle-containing macrophages were identified in the MLN, but only after 6 months of carbon ingestion were small numbers of particles seen in the liver and lungs. These findings have been confirmed recently using fluorescent particles (1.8 μm),[34] where despite extensive uptake into PP and the MLN, only trace amounts of particles were found in the liver and none was observed in the kidney or spleen.

The studies of Eldridge et al.,[33] Jani et al.,[35] and Ebel[36] are in general agreement about particle fate, with intestinally-derived particles being transported in the mesenteric lymph and readily identified in the MLN. These workers also reported the presence of appreciable numbers of particles in the spleens of experimental animals. Only Jani et al.[35] examined particle uptake by the liver and appreciable numbers of the particles were found. Interestingly, both Ebel[36] and Eldridge et al.[33] described a size-dependent distribution of particles after uptake. Particles less than 5 μm were found in the MLN and spleen, but particles greater than 5 μm did not penetrate further than the PP. Jani et al.[35] reported that particles of 3 μm were not taken up by PP, but this study was performed in rats, while those of Eldridge et al. and Ebel were performed in mice. Hence, the extent of particle transport to deeper body sites after uptake across the intestine generally appears to be size dependent, with smaller particles being found more widely distributed.[5]

IV. DELIVERY SYSTEMS TO PROMOTE ABSORPTION AFTER ORAL ADMINISTRATION

A range of delivery systems including particulates, lipid-based systems, and mixed micelles, have been used in attempts to promote the oral absorption of drugs that are normally poorly absorbed. However, it seems unlikely that any of these delivery systems would be capable of directly promoting lymphatic absorption, rather than simply promoting a general increase in bioavailability by some other mechanism. As was stated in the introduction, most orally absorbed drugs will be transported almost exclusively by the portal circulation, unless selective uptake into the lymphatics occurs. Few drugs are sufficiently lipophilic to be absorbed into the lymphatics, although lymphatic transport of particulates after intestinal uptake has been directly observed.[35]

A. PARTICULATE DELIVERY SYSTEMS

1. Delivery of Drugs

Hexylcyanoacrylate nanoparticles (200 nm) have been shown to be capable of enhancing the bioavailability of vincamine after oral administration[46] and poly-isobutylcyanoacrylate nanocapsules have been successfully used to orally deliver entrapped insulin.[19] A previous attempt to promote the oral absorption of insulin by adsorption to nanoparticles had proven unsuccessful,[47] but limited success had been achieved using nanoparticles (200 nm) prepared directly from insulin.[48] However, in all of these studies, neither the sites nor the mechanism of uptake of the particles were identified.

Greater success has been achieved using particulates as antigen, rather than drug delivery systems, probably as a result of the low efficiency of uptake of the particles. Only a small amount of antigen is usually needed to induce an immune response, particularly on repeated exposure, while relatively large amounts of drugs are normally required to induce a physiological response.

2. Delivery of Antigens

It has been known for many years that the nature of an antigen affects the response following oral administration, with particulate antigens generally inducing better immune responses. This was demonstrated experimentally by Cox and Taubman[49] who induced enhanced secretory immune responses to a soluble antigen following adsorption to a particulate carrier (1 to 3 μm). It was postulated that the enhanced immune responses resulted from the greater ability of the particulates to gain access to the PP.[49] More recently, three papers have described the use of microparticles as antigen delivery systems for oral immunization.[33,50,51] The particulate carrier systems induced enhanced secretory immune responses to both entrapped[33,51] and adsorbed antigens[50] and also induced enhanced systemic immune responses.[33,50]

B. LIPID-BASED DELIVERY SYSTEMS

1. Liposomes

Since the early 1970s, liposomes have been extensively investigated as potential oral drug delivery systems (particularly for insulin) and the available data have been recently reviewed by Woodley.[52] Very variable and often contradictory results have been obtained by different research groups, partially as a result of the different methods of liposome preparation used and partially because of the many different compositions of phospholipid/lipid employed. Nevertheless, liposomes are generally unstable in the GIT and it was concluded that they were not really appropriate for oral delivery of insulin.[52] Furthermore, following detailed *in vitro* and *in situ* studies on liposomes in the GIT, Chiang and Weiner[53] concluded that intact liposomes were not taken up by epithelial cells of the GIT and that liposomal entrapment did not facilitate the transport of nonabsorbable drugs and prevented the transport of absorbable ones. The limited success achieved in some studies involving oral administration of liposome-entrapped insulin may be ascribed to the ability of the delivery systems to protect the hormone from degradation in the GIT. Consequently,

there seems to be little advantage to be gained by the use of liposomal delivery systems for oral administration. Alternative delivery systems such as emulsions, which are easier and cheaper to prepare, are more stable on storage and probably more effective.

2. Emulsions

It has long been known that the oral absorption of lipid-soluble agents such as sulphonamides[54] and indoxole[55] can be enhanced by administration in an emulsified form. Emulsions have also been used to promote the oral absorption of macromolecules such as insulin[56] and heparin,[57] though with limited success. The use of w/o/w double emulsions for oral insulin delivery was first described by Engel et al.[56] and further developed by Schichiri et al.[58] However, although promising results were obtained in studies in animal models, the oral insulin preparation was a less than satisfactory substitute for parenteral insulin.[58]

Mizushima and co-workers[59] described the use of "lipid microspheres" (LM) as drug carriers and these systems have been successfully employed to promote the lymphatic delivery of cyclosporin following oral administration. The LM were prepared using a French cell press and are perhaps better described as lecithin-dispersed o/w emulsions. An alternative paper describing the oral delivery of cyclosporin from emulsions has indicated that the bioavailability of the drug can be improved through the reduction of droplet size.[60] Enhanced absorption from the emulsion with the smaller droplet size was thought to be due to the greater surface area of the dosage form, leading to the formation of more micelles in which the cyclosporin was solubilized. Increased surface area of the dosage form and resultant enhanced solubility of cyclosporin may also partially explain the effect of the LM on absorption.[59] However, it was claimed that LM were endocytosed intact by epithelial cells of the GIT in the same way as liposomes, although no evidence was offered to support this claim.[59]

The ability of lecithin-dispersed aqueous preparations containing medium chain triglycerides to promote the lymphatic absorption of d-α-tocopherol (vitamin E) acetate following oral administration has been reported by Kimura et al.[61] It was first proposed by Bloom and co-workers[62] that lymphatic or portal absorption of a given fatty acid was dependent on its chain length and Palin et al.[63] demonstrated the effect of fatty acid chain length on absorption of lipophilic drugs. Similiarly, Takada et al.[64] have reported enhanced bioavailability of colony stimulating factor in rats following intraduodenal administration in a 10% solution of a commercially available castor oil derivative (HCO-60). The ability of various lipids to promote lymphatic transport of drugs is dealt with in more detail in Chapter 4.

An emulsion-based "novel" oral insulin delivery system has recently been described by Cho and Flynn.[65] The stated rationale was to present a chylomicron-like system to the absorptive surface of the GIT, thereby implying that the emulsion was absorbed intact. It was also stated that the system was designed so that a substantial proportion of the administered dose of the insulin would

be delivered to the liver through attachment to chylomicrons and their remnants. This is unlikely. Chylomicrons are formed by resynthesis of fatty acids to triglycerides in the enterocytes, subsequently to be transported to the general circulation via the lymph. Several specific apoproteins have a vital role in chylomicron transport and remnant metabolism and it is not clear how insulin could remain attached to a lipid particle through the various stages of metabolism and modification. An alternative explanation for the reported effects is that the formulation (water-in-oil emulsion plus enzyme inhibitor) affords good protection of the drug and that the incorporated nonesterified fatty acids act as so-called soaps in the alkaline intestine, thereby modifying membrane permeability.

Hence, although emulsions, double emulsions, and liposomes have all shown some success as oral drug delivery systems, there remains little convincing evidence to suggest that such systems are taken up intact and transported lymphatically.[66]

C. MIXED MICELLES

The use of lipid-surfactant mixed micelles (MM) to promote intestinal and rectal absorption of macromolecules and small molecular weight drugs has been pioneered by Muranishi and is discussed in Chapter 7. MM are reportedly safe and effective absorption enhancers for many drugs and are particularly effective following rectal administration.[67] The ability of MM to act as absorption enhancers for small particulates delivered into the large intestine has also been reported by Muranishi et al.[68] These studies employed particulate inks and colloidal gold as markers and it was concluded that the upper limit for particles absorbed by the large intestine under the influence of MM was 40 nm. The absorbed particulates were selectively transported in lymphatics and accumulated in regional lymph nodes. The mechanism of uptake was unknown, but openings in the tight junctions of the rectal epithelium were identified and it was in the rectum that the MM were most effective. Concerns have been expressed about the safety of various penetration enhancers, particularly those used rectally, and the possible consequences of co-absorption of toxic compounds along with drugs cannot be ignored. Perhaps penetration enhancers capable of promoting absorption of particulates as large as 40 nm should be used with caution.

V. LYMPHATIC DELIVERY OF PARTICULATES BY THE PARENTERAL ROUTE

A. FACTORS CONTROLLING THE UPTAKE OF PARTICULATES INTO THE LYMPHATICS AFTER PARENTERAL ADMINISTRATION

Locally injected particulates can be taken up into the lymphatic system via the lymphatic capillaries (initial lymphatics). Electron-dense tracers have been used to show that the major pathway by which this occurs is the intercellular

junction, although transport through the cell in vesicles by pinocytosis can occur from both the luminal and connective tissue surfaces.[69]

The mechanism of intercellular uptake is still in dispute. Leake and Burke[70] suggested that the anchoring filaments and overlapping cell structures of the lymphatic capillaries provided the basis for active filling due to pressure differentials. Increased fluid within the interstitium was thought to force the surrounding collagen bundles apart, in turn exerting tension on the anchoring filaments which pull open the vessel walls. Nevertheless, Casley-Smith et al. challenged this theory, since they could only observe the mechanism occurring in edematous tissue.[71]

Alternative mechanisms include the "lymphatic suction theory" which proposed that suction of fluid into opened lymphatic capillaries was followed by contraction of the endothelial cells or external compression. This resulted in the propulsion forward of the capillary contents.[72] The "osmotic pull hypothesis" proposed that compression of the lymphatic capillaries caused a secretion of water, resulting in an increase in the concentration of luminal protein, which set up an osmotic pull upon relaxation.[73]

The rate of particulate uptake from an interstitial injection site is dictated by its rate of diffusion through the interstitium. Consequently, the rate of uptake depends upon the physiological state of the interstitium, e.g., the presence of inflammation or edema, the anatomical site of injection, and also on the physiochemical properties of the particle. The importance of these properties are discussed here.

1. Size

The major factor determining the behavior of particulates after interstitial injection is size.[74] Small diameter particles (less than a few nanometers) are usually exchanged via the blood capillaries whereas larger particles, up to about 100 nm, are selectively transported into the lymphatic capillaries. Particles over a few hundred nanometers in size will be trapped in the interstitial space for a longer time. This size-dependent transportation has been demonstrated in both animals[75] and humans,[76,77] in studies using standard scintigraphic colloids. The effect has also been shown for liposomes, following intramuscular (IM)[78] and subcutaneous (SC)[79] injections. The larger liposomes remained at the injection site for longer periods than the smaller ones. However, for intraperitoneal (IP) injections, size had no effect[80] since uptake is not dependent upon diffusion through the interstitial space, but occurs from a cavity.

2. Number of Particles

The number of injected colloid particles has also been reported to influence the rate of outflow from the injection site.[81] For liposomes, decreasing the total amount of lipid injected resulted in greater lymphatic uptake.[78]

3. Surface Charge

Alteration of liposomal composition has been shown to change the rate of absorption from IP injection sites.[82] This appears to result from changes in

surface charge, with negative liposomes (incorporating dipalmitoyl phosphatidic acid in their lipid phase) being taken up faster than those with a positive charge (containing stearylamine). The half lives at the injection site were approximately 5.1 and 9.7 hr, respectively, for 200 nm liposomes.

4. Molecular Weight

The molecular weight of compounds is also known to affect their lymphatic absorption. Recently, Supersaxo et al.[83] have shown in sheep that for SC-injected water soluble macromolecules, there is a positive linear relationship between the molecular weight of the macromolecule and the proportion absorbed by the lymphatics. However, for particulates, size, rather than molecular weight, is more likely to be the rate-determining factor.

B. THE DISTRIBUTION OF PARTICULATES IN THE LYMPHATICS

Particles are propelled through the lymphatic system by the muscular contractions of the vessel walls and the action of valves. Upon reaching a lymph node, they can be trapped by one of two mechanisms: (1) simple mechanical filtration, or (2) phagocytosis by the reticulo-endothelial cells. Diverging opinions exist as to the effectiveness of the filtration mechanism. Overall, it is thought that the nodes provide a high degree of retention, but not an absolute barrier. There appears to be no complete saturation of a node before uptake into successive nodes occurs.

1. Depth Filtration

The filtration mechanism of nodal particulate trapping is thought to resemble that of a depth filter,[84] in which particles are brought close to the filter (nodal structures) by Brownian motion and flow changes, and become immobilized onto the node by mutually attractive forces. Depth filtration efficiency is known to decrease with increased flow rate and increase with increases in size or density of the particles. For a given colloid, each filter layer extracts the same proportion of particles in a given time (assuming a uniform bed and flow rate).

2. Phagocytosis

The other model of colloid retention in lymph nodes is the biological model of phagocytosis. Receptors on macrophages in the lymph nodes recognize particles either by the nature of the particle itself or through opsonic coating.[85] Attachment and engulfment of the particle then takes place. The phagocytic response of the cell membrane is believed to be proportional to the size and number of particles presented to it.[86,87] It is also possible to define a constant index of phagocytosis, K, for a given node, although its value increases with increasing particle size.[84] A "critical colloid dose" above which uptake becomes a measure of phagocytic capacity also exists.[88]

The surface characteristics of the colloid, e.g., charge and surface coatings,

also influence the extent of phagocytosis,[85] as they affect the interactions between the colloid and macrophage. For example, small unilamellar negatively-charged liposomes are localized in lymph nodes to a higher degree than positively-charged liposomes, and positively charged much more than neutral liposomes.[89] Hydrophobicity may also play an important role, as it has well documented effects on the adhesion properties and susceptibility to phagocytosis of bacteria.[90,91]

3. Optimization of Particulate Delivery to the Lymphatics

It would therefore appear that optimization of particulate uptake into lymph nodes (if this is the desired goal) involves achieving a delicate balance between the various physiochemical properties of the particle. Although the number of particles, their composition, charge, and hydrophobicity all have an effect, the major controlling factor appears to be size. Small particle size optimizes uptake into the lymphatic capillaries whereas larger particles show enhanced lymph node uptake (retention), either by filtration or phagocytosis.

Strand and Bergqvist concluded from their studies on standard scintigraphic colloids that the optimum size for primary lymph node retention is between about 10 to 50 nm (Figure 4).[92] However, these values may differ for alternative delivery systems.

VI. OPPORTUNITIES FOR SITE-SPECIFIC DELIVERY IN THE LYMPHATICS USING COLLOIDAL CARRIER SYSTEMS

In general, the types of colloidal systems employed in targeting to the lymphatics can be classified into two groups: (A) classical radiocolloids used for lymphoscintigraphy and other diagnostic purposes, and (B) drug delivery systems formulated specifically to transport drugs to desired sites within the lymphatics. These will be discussed below, the latter in more detail.

A. LYMPHOSCINTIGRAPHY USING RADIOCOLLOIDS

Gamma scintigraphy of the lymphatics (lymphoscintigraphy) by SC administration of a suitably radiolabeled tracer affords an opportunity to investigate the filtering function of lymph nodes.[93] The colloid is usually injected interstitially, at the most distal anatomical site at the origin of the lymphatics under study, or close to the site of a primary lesion.[92] It was originally thought that absence of colloid accumulation in a lymph node could be considered indicative of malignant invasion, presumably due to blockage by tumor cells. However, as discussed below, this remains unproven.

Sherman et al.[94] first reported the selective lymph node migration of interstitially-injected ^{198}Au (gold)-colloid in 1950. The small size range of 5 to 10 nm yielded high uptake levels. Since then, many other colloids have also been investigated for lymphatic uptake. Technetium-99m soon replaced ^{198}Au as the radionuclide of choice, due to its optimum photon energy, which led to

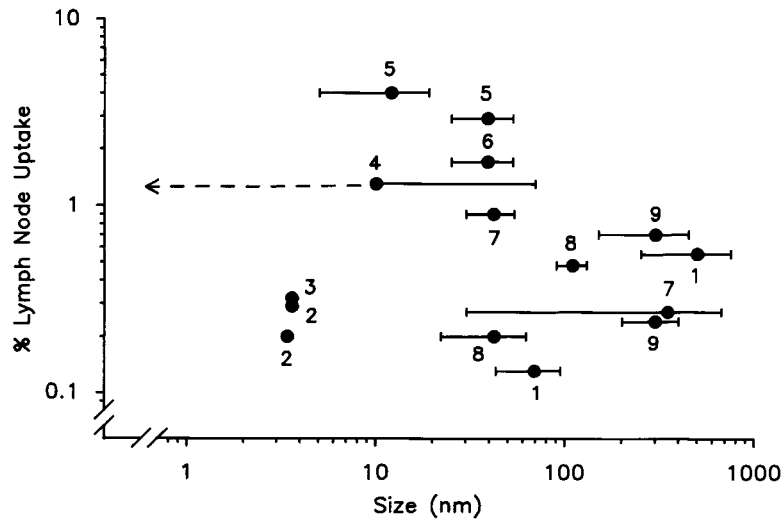

FIGURE 4. Parasternal lymph node uptake of radiopharmaceuticals 2 hr after SC bilateral injection near the xiphoid process in rabbits. Horizontal lines represent particle size ranges and the symbols represent the median particle size. Key: 1 = $^{99}Tc^{m}$–μAA; 2 = $^{99}Tc^{m}$–HSA; 3 = $^{99}Tc^{m}$–Antibodies; 4 = $^{99}Tc^{m}$–mμAA; 5 = ^{198}Au; 6 = $^{99}Tc^{m}Sb_2S_3$; 7 = $^{99}Tc^{m}$(Re)S; 8 = $^{99}Tc^{m}$(Sn)S; 9 = $^{99}Tc^{m}{}_2S_7$ (From Strand, S.-E. and Bergqvist, L., *Crit. Rev. Ther. Drug Carrrier Sys.*, 6, 211, 1989. With permission.)

a lower absorbed dose and resulted in reduced local tissue necrosis. The use of $^{99}Tc^{m}$-sulfur colloid was first suggested in 1973,[95] although the efficiency of this material was found to be highly dependent on the size range used,[76,96] smaller sizes promoting lymphatic uptake.

The most popular colloid for lymphoscintigraphy to date is technetium-99m-antimony sulphide colloid ($^{99}Tc^{m}Sb_2S_3$ or $^{99}Tc^{m}$ASC). It was developed by Garzon et al.[97] as a liver scanning agent, and has a size range of 5 to 15 nm. Studies in tumor-bearing rabbits[98] and patients with breast carcinoma[99–101] showed a reduction in colloidal uptake into regional lymph nodes compared to controls. However, studies in patients with malignant trunk melanoma revealed high uptake levels into a significant number of metastatic nodes.[102–104] The unpredictability of uptake into metastatic nodes has been discussed by Strand et al.[105] However, lymphoscintigraphy is still believed to be useful for elucidation of the direction of lymph flow from the primary tumor site (for surgical, radiation, and follow-up information) and for lymphatic system function studies. When compared to other colloids used for lymphoscintigraphy, $^{99}Tc^{m}Sb_2S_3$ was shown to give the highest nodal uptake levels,[74,75,86,106] probably due to its optimum size.

B. PARTICULATE DELIVERY SYSTEMS

1. Colloidal Carbon

Colloidal carbon has long been known to be absorbed into the lymphatic system to a greater extent after extravascular injection than after IV injec-

tion.[107] It has been shown to appear predominantly in the sinus, follicles, and paracortex of the regional lymph nodes. The particles are extensively phagocytosed in the medullary sinus, and the lymphendothelium also forms a barrier to diffusion.[108] Little phagocytosis occurs in the subcapsular sinus.

More recently, a new lymphatic drug delivery system for injection into tissues has been investigated, comprising various anticancer drugs adsorbed to colloidal carbon particles of 200 nm or less.[109] After injection into the subserosa of the gastric wall of dogs, lymph node levels of drug were greatly increased (approximately 100 times) over the levels obtained with solutions. Furthermore, the LD_{50} values were higher. In patients, 33% of lymph nodal metastatic lesions became degenerative or inflammatory after a single administration of mitomycin C (MMC) adsorbed to carbon particles.

The particles also showed some selectivity for metastatic over normal nodes[109,110] since they were shown to preferentially adhere to malignant cells.[110] The system also had slow-release characteristics, as the adsorbed drug and free (released) drug are in a dynamic equilibrium, represented by the adsorption isotherm.[110]

For drugs such as bleomycin and peplomycin, the dose-limiting factor is the development of pulmonary fibrosis. Carbon adsorbed systems for IP and IM delivery of these drugs have so far shown zero incidence of pulmonary toxicity in mice.[111] Colloidal carbon systems therefore appear to show promise as lymphatic drug delivery systems, and have been reviewed in more detail elsewhere.[112]

2. Lactic Acid-Oligomer Microspheres

L-lactic acid-oligomer microspheres containing aclarubicin (ACR) can achieve sustained release of ACR *in vitro*, the rate of release being dependent on the molecular weight of the oligomer used.[113] These microspheres have the advantages of good stability, nontoxicity, efficient drug loading, and biodegradability. Microspheres formed from L-lactic acid-oligomer with an average molecular weight of 3600 have been targeted to the lymphatics.[114] Following IP injection to rats, two sizes of microspheres (less than 1 μm, and 1 to 5 μm) both yielded approximately constant levels of ACR in thoracic lymph over 10 days. The larger particles yielded higher levels of ACR in thoracic lymph. However, this may be attributed to a higher initial drug loading of the larger particles, and not an increased lymphatic uptake.

C. LIPID-BASED DELIVERY SYSTEMS

1. Liposomes

Since their invention by Bangham et al.[115] in the mid-1960s, liposomes have been used extensively in attempts to target anticancer compounds more effectively to the lymphatic system. The structure and kinetics of liposomes, as well as the rationale for their use in cancer chemotherapy and the types of problems encountered, have been well reviewed by Weinstein.[116] The major problems encountered with liposomes are instability and disruption by serum components (many of which are found in lymph).

The first observation of interstitial targeting of liposomes to lymph was made by Ryman et al.[117] when they reported uptake into the rat axillary lymph nodes of an intravenous injection which had accidentally been administered interstitially. Since then, many local parenteral routes of administration have been investigated for the lymphatic uptake of liposomes.

The intramuscular route was chosen by Jackson,[78] since it had been previously suggested that the lymphatics show increased permeability in regions of high muscular activity.[118] The effects of the physicochemical properties of liposomes containing ^{3}H-inulin on their lymphatic uptake after IM injection in rats was investigated.[78] Using liposomes of identical composition but of two different size ranges (0.3 to 2.0 μm, and 0.15 to 0.7 μm) it was shown that the smaller liposomes gave lower values for retention at the injection site and higher values for lymph node levels of inulin. Since then, Ohsawa et al.[119] have shown that the kinetics of liposome uptake from IM sites is also dependent on the method of preparation and presumably the composition of liposomes.

The SC route has been used more extensively for the lymphatic targeting of liposomes. In the early 1980s, Khato et al.[120] studied the activity of melphalan (MPL) entrapped in liposomes against lymph node metastasis in rats. Initially, small neutral liposomes containing tracer amounts of ^{14}C-MPL and ^{3}H-PC (phosphatidylcholine) were injected SC into the thighs of rats.[120] The results demonstrated a more sustained concentration of ^{14}C activity in the regional lymph node for liposomal MPL than was found with free MPL, and prolonged retention at the injection site. Furthermore, a greater reduction in metastatic lymph node size at a fourfold lower dose than that of the free MPL was observed for liposomal MPL. A later study also demonstrated that the ^{14}C and ^{3}H levels in ipsilateral lymph nodes were "strikingly proportional" from 4 to 24 hr after dosing, suggesting that MPL was absorbed via the lymphatics in association with liposomal lipid.[121]

The effect of liposomal particle size on lymphatic uptake was subsequently investigated.[79] Small uniform liposomes (mean size 34 nm measured by electron microscopy) gave high sustained levels in regional lymph nodes, whereas larger polydisperse liposomes (1.8 to 2 μm) remained longer at the injection site. Kaledin et al.[122] also demonstrated rapid uptake from SC injection sites in mice using small liposomes (mean size 43 nm), and a 5- to 50-fold increase in the popliteal lymph node level of triiodothyronine compared to free drug.

The effect of liposome size on lymphatic uptake from SC sites in the rat was also investigated by Tumer et al.[123] Using liposomes of sizes 30 to 60 nm and around 400 nm, it was again demonstrated that smaller liposomes show a faster rate of uptake from the injection site than larger liposomes, resulting in higher levels in the primary and secondary nodes in this study. However, the smaller liposomes also showed higher levels in the circulation. Thus, although the larger liposomes showed lower values for lymphatic uptake and nodal trapping in normal rats, they had the advantages that levels of uptake were

increased in tumor-bearing animals and only negligible amounts reached the circulation.

Perez-Soler et al.[124] demonstrated that in the dog, SC uptake of large, negative multilamellar vesicles (MLVs) could be nearly limited to the popliteal primary nodes if divided multiple doses were administered. They also suggested that MLVs had the advantages over SUVs (small unilamellar vesicles) of a slower, more prolonged delivery, greater stability, lower systemic distribution, and increased ability to entrap drugs.

Osborne et al.[125] investigated the ability of labeled liposomes with different charges to detect cancer metastases in lymph nodes. Monitoring of $^{99}Tc^{m}$-labeled liposomes after interstitial (IS) injection into the rat foot pad originally showed that positively-charged and neutral liposomes were taken up more extensively than negatively-charged liposomes into normal primary nodes, and that positively-charged liposomes showed a less marked suppression of uptake in the presence of metastases. However, these results were later invalidated when the concurrent use of an aqueous phase marker, ^{125}I-PVP, showed that the technetium marker did not represent intact liposomes in lymph nodes.[126] Uptake of ^{125}I showed that the order of liposome localization in normal rat nodes was actually negative > positive > neutral. It was also shown that although most liposomes were structurally intact after 5 hr, small molecular weight substances such as methotrexate leaked from them, suggesting a possible lymph-induced pore formation which may be useful for controlled drug release.

It has also been suggested that antibodies, particularly monoclonal, can be attached to liposomes by various methods in order to target to cell types more effectively.[127] Mangat and Patel[128] have shown that after foot pad injection in rats, positively- and negatively-charged liposomes with noncovalently bound IgG showed an increased lymphatic uptake and an increased accumulation in nodes when compared to uncoated liposomes (anionic > cationic). This uptake appeared to be inhibited by free IgG.

Metastatic lymph nodes in rats have also been treated with a combination of hyperthermia and temperature-sensitive liposomes containing bleomycin.[129] An increased activity of bleomycin was seen both *in vivo* and *in vitro* compared to both free bleomycin and bleomycin delivered in a standard liposomal formulation. Work has also been conducted on the IP administration of liposomes for lymphatic delivery. Parker et al.[130] investigated this route, since particulate matter is known to be cleared from the peritoneal cavity primarily by the lymphatics, draining to the thoracic and renal nodes. It was shown that the entrapment of ^{14}C-adriamycin (ADR) in liposomes resulted in 6% of ADR appearing in lymph over a 24-hr period compared to only 1% of free drug after IP administration.[130]

The biodistribution of cytosine β-D-arabinofuranoside, which is usually well absorbed, rapidly excreted, and does not bind readily to tissues, was also altered by IP administration in liposomes.[131] Liposomal entrapment was found to decrease vascular absorption rates and to increase lymphatic uptake tenfold.

Local node levels were 300 to 1000 times higher than those for free drug at 24 hr, and the rate of metabolism was decreased. In total, over 20% of absorbed drug was recovered in thoracic duct lymph.[132]

Uptake of liposomes containing the fluorescent dye carboxyfluorescein (CF) from the peritoneum has also been demonstrated, while free CF injected IP and liposome-CF injected intraduodenally showed no lymphatic uptake, being cleared instead by the portal circulation.[133]

Further work on uptake from the peritoneum was also conducted by Hirano and Hunt.[80,82] They considered the effects of liposome composition and size using ^{14}C-sucrose as the aqueous phase model drug. Negatively-charged liposomes (containing dipalmitoyl phosphatidic acid in their lipid phase) were shown to yield the greatest retention values in nodes, correlating with their greater stability.[82] Decreasing the size of liposomes (in the range 460 to 48 nm) resulted in an increased lymph to blood ratio, a greater recovery from thoracic lymph, a decreased retention in nodes, and no change in absorption.[80] Liposomal stability also increased with decreasing size.

Table 3 summarizes the studies on liposome targeting to the lymphatics discussed in this chapter. For further information, an excellent review on the fate of liposomes in the lymphatics has recently been published by Patel.[89] Aspects of targeting to lymph nodes, leakage from liposomes, the use of effective liposomal markers and lymphoscintigraphy are all discussed in some detail. The application of liposomes in cancer chemotherapy has also been recently reviewed.[112]

2. Emulsions

Emulsions have long been considered as carriers for drug molecules and have been investigated as lymphatic targeting systems. Takahashi et al. showed that incorporation of ^{3}H-5-fluorouracil (5-FU) into various emulsions resulted in rapid uptake and long residence of the drug in local nodes after intratesticular injection into rats.[134] The order of uptake seen was water in oil in water emulsion (w/o/w) > water in oil emulsion (w/o) > oil in water emulsion (o/w) > free aqueous drug, with w/o/w levels being 9.1 and 7.9 times higher than IV administration and aqueous levels, respectively. The suggested reason for this was that as 5-FU (like most anticancer drugs) is a water soluble compound with a low molecular weight, it is surrounded by the oil phase in w/o and w/o/w type emulsions. This facilitates its uptake via the lymphatics, possibly by a similar mechanism to that involved in fat uptake from the intestines. Clinical trials of locally injected 5-FU containing w/o and w/o/w emulsions in eight patients with skin carcinomas showed an enhanced chemotherapeutic effect of the anticancer agent on lymph node metastasis.[135]

Investigations of the lymphatic uptake of mitomycin-C (MMC) and bleomycin in emulsions were conducted by Nakamoto et al.[136,137] It was demonstrated that increased drug delivery to nodes was obtained, in the order w/o emulsions > o/w emulsions, when compared to free drug levels. The IP administration route yielded higher nodal levels than IM administration, and

TABLE 3
Summary of Major Studies Conducted on Liposome Targeting to the Lymphatics

Parenteral route	Animal model	Tumor present	Drug or (aqueous marker) used	Variables investigated	Ref.
IM	Mouse	No	(Inulin)	Size and amount of lipid	78
	Rat	No	(Inulin)	Preparation method	119
IP	Rat	No	(Sucrose)	Size	80
				Composition/ charge	82
			Adriamycin	—	130
			Cytosine b-D-arabino-furanoside	—	131, 132
			(Carboxy-fluorescein)	—	133
SC	Rat	No	Melphalan	Size	79
		No	(PVP)	Charge and carrageenan effects	126
		Carcinoma 13762	Melphalan	—	120, 121
		Walker 256	Bleomycin and (carboxy-fluorescein)	Size and composition	123
		Rd/3 or inflam-mation	—	Charge	125
	Mouse	No	(triiodothyronine)	—	122
	Mouse and rat	No	(PVP)	Charge and IgG coating	128
	Rat	AH66 ascites	Bleomycin	Temperature sensitive	129

bleomycin gave lymph node levels greater than those obtained with MMC. It was also shown that incorporation of gelatin into the o/w emulsions resulted in some binding of MMC to the oil phase and yielded an increased uptake of the drug into lymph nodes. It was therefore suggested that it was the interaction of the drug with oil which facilitated lymphatic absorption,[138] although the reason could be the decrease in emulsion droplet size (from 5 to 6 μm to 2 to 3 μm) on addition of gelatin.

The incorporation of gelatin into a w/o emulsion was shown to induce the delivery system to behave *in vivo* as a microsphere in oil emulsion (s/o), and to further enhance the uptake into the lymphatics of an aqueous phase marker from IM sites in rats.[138] A similar enhancement of the lymphatic uptake of 5-

FU[139] and bleomycin[140] was also observed with the s/o emulsion system. When an S/O emulsion containing bleomycin was injected into the appendix wall of rabbits with a local carcinoma which was known to metastase to regional nodes, the survival time was lenghthened to about twice that of the control.[141]

The above studies all considered the effect of emulsion composition on lymphatic uptake. The effect of size may also be important. For example, after IP injection of iodinated emulsions, the opacification of mediastinal lymph nodes was shown to occur within hours for "fine" emulsions (4 μm), but took days for "coarse" emulsions (15 μm).[142] Injection volume and massage of the injection site have also been shown to affect the rate of lymphatic uptake.[143]

Much of this work has been previously reviewed in some detail by Davis et al.[144] as has the application of emulsions to cancer chemotherapy.[145] Overall, the IM,[136–140,146] SC,[134,141] and IP[136,137,147] routes (summarized in Table 4) for emulsion delivery to the lymphatics appear to yield very variable results, being highly dependent upon both the site of administration and the properties of the emulsion system itself. Although increased lymphatic levels compared to free drug are often observed, the actual lymph node levels obtained are still comparatively low.

VII. SUMMARY AND PERSPECTIVES

In view of the many reports from a number of independent workers describing the uptake of a range of particulates across the GIT, it must be concluded that intestinal uptake of particulates is possible in certain circumstances. However, whether intestinal translocation of particulates is merely an interesting experimental phenomena or an important physiological mechanism with potentially damaging consequences remains unproven. Demonstrations of uptake have been largely confined to small laboratory animals in "nonphysiological" experimental situations. Whether uptake across a normal unobstructed intestine occurs in larger species such as man remains unproven. Nevertheless, the available evidence suggests that humans possess PP which are fundamentally similiar to those found in rodents, with M cells found dispersed among the ordinary cells of the FAE.[148]

Even in those species which have been extensively studied, the qualitative and quantitative nature of particulate uptake remains poorly defined. Often contradictory and confusing findings are reported. For example, in a study in a closed gut loop system in the rabbit, which has a relative abundance of M cells in the FAE, 5% of an administered dose of microparticles were reportedly taken up across the GIT.[43] Such a high degree of uptake would not be expected to occur in studies involving an unobstructed intestine in a species with much fewer M cells, e.g., the rat. However, some workers have claimed a degree of uptake of much greater than 5% into rats, following oral administration.[37–39]

Nevertheless, the majority of available evidence indicates that particle uptake across the GIT occurs to only a very limited extent.[5] This is perhaps not surprising, since the implications of extensive particle uptake would be a cause

TABLE 4
Summary of Major Studies Conducted on Emulsion Targeting to the Lymphatics

Parenteral route	Animal model	Tumor present	Drug or (marker) used	Emulsions or properties investigated	Ref.
Testicular	Rat	No	5-Fluorouracil	w/o, o/w, w/o/w	134, 135
IM/IP	Rat	No	Mitomycin C	w/o, o/w and emulsifier effects	136
			Bleomycin	w/o, o/w and spray-dried emulsion	137
IM	Rat	No	(Iodohippuric acid)	w/o, o/w, s/w	138
			5-fluorouracil	w/o, o/w	139
			Bleomycin	w/o and complexation with dextran	140
			—	w/o, s/o and effect of surfactants, injection volume, and massage	143
			Interferon	—	146
IP	Rat	No	Cyclosporin A	Mixed micelles	147
	Rat and rabbit	No	—	Effect of size	142
Tumour + local (SC)	Rat	Hepatoma AH-66	Bleomycin	w/o	135
Local (SC)	Man	Various	Bleomycin/ Mitomycin C	w/o, w/o/w	135
Appendix wall	Rabbit	VX2 carcinoma	Bleomycin	w/o, s/o	141

for much concern which would probably have been previously highlighted. Consequently, it appears likely that only very potent drugs with a wide therapeutic index could be considered for oral delivery as particulate delivery systems. Predictablity of the absorbed dose appears difficult and poor clinical control or the induction of dose-related adverse effects are therefore likely. In addition, the possibility of adverse effects or toxicity would be greatly enhanced by acute or chronic inflammatory intestinal diseases, which could promote the extent of uptake of the delivery system. Although oral drug delivery options with particulate carriers appear limited, one should not overlook the successes already achieved (Section IV A.1). Some success was achieved using nanocapsules to deliver the important peptide drug insulin,[19] although aspects of this study remain poorly explained. However, more success has been achieved using microparticles as antigen delivery systems for oral immunization (Section IV A.2). This is not surprising since the PP are the sites of induction for the secretory immune response[8] and are also the sites of maximal particle uptake.[5] Furthermore, much smaller amounts of proteins are generally required to induce an immune response, relative to the amount normally needed to induce a physiological response. Systemic immune responses are also induced by oral immunization with microparticles[33,50] since once taken up, particles are transferred to the MLN and possibly further.[5]

Hence, particulate carriers seem to have considerable potential as oral antigen delivery systems, but this line of research is very much in its infancy. In addition, particulate carriers may have some potential as oral drug delivery systems, although the data are less convincing and there are considerable problems. Those studies describing uptake of particles following a single oral dose[20,33] are particularly encouraging. Nevertheless, few drugs would appear appropriate for oral delivery by particulates because of the erratic and unpredictable nature of uptake. Perhaps future studies will address this ill-defined issue of the reproducibility of uptake on repeated administration. If particulates are to be seriously considered for oral drug delivery, then it is better to achieve low yet reproducible uptake, rather than much greater yet unpredictable uptake.

The data on the use of lipid-based delivery systems for oral administration of small molecular weight drugs are quite encouraging and are dealt with more extensively in Chapter 4. However, the use of lipid-based delivery systems for the oral delivery of macromolecules is much less encouraging. Only limited success has been achieved over the years despite many attempts to deliver drugs such as insulin and heparin in liposomes, emulsions, double emulsions, and various alternative carrier systems. The rationale for the use of many of these systems seems flawed, since it is well acknowledged that such systems cannot penetrate the GIT intact. Consequently, there seems little point in constructing complex delivery vehicles that will not survive in the intestinal environment long enough to achieve any beneficial effect. However, macromolecules are absorbed intact across the GIT to a limited degree under normal circumstances and it may be possible to promote this absorption. Several lipid-based oral delivery systems have shown some success, probably as a result of

their ability to protect the co-administered macromolecule from degradation or to enhance the permeability of the intestine. The results described by Cho and Flynn[65] involving oral delivery of insulin in three experimental subjects are promising. Their delivery system may offer greater protection against degradation combined with intestinal permeability enhancement. However, their findings need to be substantiated by further experimental data and their delivery system currently remains of unproven efficacy.

The problem posed by the local parenteral delivery of particulates to the lymphatic system is less complex than that of oral delivery in that the process exploited is the normal physiological action of the lymphatic system, that of clearing fluid from the interstitial space. However, the exact mechanisms involved are still under debate.[70–73] As discussed in this chapter, many groups of workers have attempted to target parenterally to the lymphatic system using a variety of particulate systems. In general, however, the studies conducted have not adequately controlled the physicochemical properties of the particulate and formulation employed which, on the occasions when they have been considered, have been shown to drastically affect lymphatic fate. The major influencing property was found to be particulate size,[75–79,123,142] although charge,[82,125,126,128] concentration,[78,81] and the volume of the formulation injected[143] also affected lymphatic uptake and transport.

Lack of consideration of physicochemical effects is most evident in the published work employing emulsion systems as targetable carriers. Typical studies have investigated the effect of changing the composition of a drug-containing emulsion on its lymphatic uptake from various parenteral sites in animal models (Table 4). However, a large proportion of these studies did not quote the size of the emulsion droplets employed,[134,135,139,140,146] even though size has been shown to affect the lymphatic uptake of emulsions.[142] When particle size was quoted,[136–138] the size ranges tended to be quite large and variation in size often existed between the emulsion systems being compared. The range of emulsion sizes used was also much larger than the "optimum" size range of 10 to 50 nm suggested by Strand et al.[76] (typically 1 to 10 μm), although it may be that the optimal size range for lymphatic delivery varies depending on the type of particulate system employed. As the studies conducted have not been adequately controlled, it is difficult to assess from current data the relative usefulness of emulsions for lymphatic targeting. Although they are taken up into the lymphatic system from local injection sites, nodal levels obtained so far have been relatively low. If the size of the emulsions could be reduced, perhaps by the production of microemulsion systems, then lymphatic levels may be increased.

Liposomes have been more thoroughly investigated as lymphatic targeting systems. Most of the studies conducted have attempted to investigate the effects of physicochemical properties on lymphatic uptake as well as the ability of liposomes to deliver specific drugs to lymph nodes. More recently, nonlipid particulate systems such as colloidal carbon[109–112] and lactic acid-oligomer microspheres[113,114] have also been developed and have shown promising levels

of lymphatic uptake and retention. One of the major problems of all the particulate systems discussed is the fact that a large proportion of the injected dose remains at the injection site. For orally delivered drugs lack of absorption does not present a large problem as the drug is quite rapidly excreted from the gastrointestinal tract. However, for locally injected drugs nonuptake into the lymphatic system results in a prolonged action of the drug in the surrounding tissue area. For cell-toxic anticancer agents such an effect would be highly undesirable.

Overall, it can be concluded that the local parenteral administration of particulates is a promising technique for achieving selective drug delivery to the lymphatic system, and has been shown to yield clinically effective results in animal model metastases. However, more basic experimental data investigating the effects of the physicochemical properties of particulates on their lymphatic distribution are required, so that a more structured approach to designing biodegradable systems can be applied in the future. Further work is also required to investigate methods of increasing the percentage of particulates taken up from the injection site.

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